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TAXONOMIC STUDY OF YEASTS

ASSOCIATED WITH FRUITS, GRAINS, & BEVERAGES

IN MASAKA, RAKAI, MBABARA, RUKUNGIRI, JINJA, TORORO & IN MBA

BY

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DEDICATION

To GNMJ and all Ugandans in whose endeavours to maintain sustainable food production and plant resources this study is directly of importance.

DECLARATION

I, the undersigned, declare that to the best of my knowledge the dissertation for the degree has not been submitted to any other University.

DATE.....

SIGN.....

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ABSTRACT

The study was undertaken during the period 1991-1994 to isolate and identify yeasts associated with surfaces of fruits, grains and fermented products of these materials.

The major aims of the study were that at the end of the research certain outputs would be realized. The following are the realized outputs as projected:

- a) Identified strains of yeasts: Schizosaccharomyces pompe, Saccharomyces cerevisiae, Saccharomyces chevalieri, Saccharomyces pastorianus, Saccharomyces ellipsoides, Debaryomyces hansenii, Dekkera bruxellensis, Lodderomyces elongisporus, Saccharomyces capensis.
Other strains coded for cluster analysis: Candida Y 42, Candida Y 50, Debaryomyces Y 46, Debaryomyces Y 42, Candida Y 33, Candida Y 6, Candida 2, Candia 11, Hansenula Y 49, Hansenula Y 62, Hansenula 62, Hansenula 32, Hanseniaspora Y 67, Hanseniaspora Y 68, Hanseniaspora Y20, Saccharomyces Y 30, Saccharomyces Y 68, Saccharomyces Y 21, Saccharomyces Y 25, Saccharomyces Y 12, Saccharomyces 30M, Saccharomyces 30 N, Saccharomyces 30 P, Schizosaccharomyces Y 56, Schizosaccharomyces Y 26, Schizosaccharomyces 44 M, Schizosaccharomyces 44 N, Schizosaccharomyces 44 T, Cryptococcus Y 57.
- b) Yeast strains whose morphological and physiological description did not confirm with available descriptions on yeasts, such as those of van der Walt and Yarrow (1984), Kreger van Rij (1984), Phaff and Starnur (1987), Barnett, Payne and Yarrow (1983) included the following strains: Y 1, Y 2, Y 4, Y 5, Y 7, Y 8, Y 9, Y 10, Y 14, Y 22, Y 28, Y 32, Y 38.
- These were fully characterised for cluster analysis (numerical taxonomy)

Identified and classified strains resulting in pure culture form a potential source for future research projects in yeast applied microtechnology and other research projects such as developing new mutants for improved yeast biosynthesis through crossing with existing useful yeast strains.

Consultancy and contractual research materials in form of stocked yeast strains are available.

Experiments have been performed as follows:

- i) Influence of Tonto on yeast growth and viability;
- ii) Ethanol tolerance in selected yeasts;
- iii) Effect of acetic acid and lactic acid on fermentation of glucose and growth of yeasts;
- iv) Changes in yeast population during fermentation;
- v) Selected yeasts for controlled aerobic and anaerobic fermentation.

Large quantities of fermented malted maize, millet, sorghum and banana juice products are consumed daily among the people in Uganda. The same products are used during funeral rites, marriages, and other cultural ceremonies.

Cassava (after detoxication) is often added to the malted millet and sorghum flour to make a dough for indigenous "bread making".

The study was also undertaken to study the yeasts responsible for certain fermentation processes. The fermented products of bananas, sorghum, millet, and cassava investigated were tonto, kwete, and ajon/amarwa made in Uganda.

In Masaka/Rakai the mean count of the colony-forming yeasts units (c.f.u.) on fruits were 74.4 cm^{-2} ; on grains 30 g^{-1} ; in fermented juice/beverage the c.f.u were $5.07 \times 10^7 \text{ ml}^{-1}$

In Mbarara/Rukungiri the counts were fruits 75.6 cm^2 ; grains 26 g^{-1} ; beverage $5 \times 10^7 \text{ ml}^{-1}$.

In Jinja/Tororo/mbale the counts were Fruits 2.5 cm^{-2} ; grains 25 cm^{-2} ; beverages $1.5 \times 10^7 \text{ ml}^{-1}$.

Experiments on selected strains of yeasts show that some of the yeasts are preferentially anaerobic and produce large quantities of gas, important in bread-making or CO_2 production. Yield of ethanol under anaerobic conditions and biomass product under aerobic conditions were verified.

There was low pH change during controlled fermentation (pH 4.0 to 3.8). The pH shifted significantly during uncontrolled fermentation typical of local brewing conditions. This indicated high level of acid production. Acetic acid and lactic acid were produced during uncontrolled fermentation by Acetobacter and Lactobacter.

Yeasts were unable to withstand toxic levels of acetic and lactic acids during prolonged fermentation.

Another good feature shown by selected yeasts is effective flocculancy which would aid in yeast harvesting for biomass production and in production of clear wine (tonto).

Pasteurization studies were conducted on 24 strains. In general the yeasts were more resistant to heat in tonto than in controls (distilled water plus glucose). Alcohol was the tonto constituent that had the greatest effect on heat resistance. D-values for all test species/strains were inversely related to the ethanol concentration. The response of yeasts to other factors such as pH and sugar content varied with the species.

Ethanol is one of the products of the metabolism of glucose sugar in tonto. The amount produced was directly related to the concentration of glucose in the medium. Some yeasts were intolerant of ethanol in their environment.

Yeasts have been discussed according to their cultural, morphological and physiological characteristics. With the knowledge so obtained, it was possible and fruitful to consider the relationship (based on vigour and pattern) of yeasts among themselves. Numerical taxonomy was applied to give a more objective system of classification of the isolated yeasts. By studying clusters, it was possible to determine the characteristics the yeasts share as well as those in which they differ.

Cluster analysis was used to identify the yeasts and their potential for biomass and ethanol yield and CO₂ production.

The word "yeast" means fermentation. The French word, levure, or the Latin word levare means to raise. The German gist or the Greek zestos refers to the bubbling foam during the process of fermentation. The ancient world of the Greek and Romans mixed fermenting juices with bread dough to manufacture raised or leavened bread.

In Uganda, fermentation is as old as the history of its tribes. Each ethnic group has its history associated with the method used to produce the "beer" or "wine" or its "bread" or "pastes".

This is shown by the important role the beverage plays in cultural activities, such as marriage, funeral rituals, harvests, ebalu (initiation ceremony) and other ceremonies.

There is plenty of unwritten literature among the people of Uganda which gives details on fermentation procedures and processes (see descriptions: Tonto, Kweto, Muramba, Ajon/Amarwa, akiria nuajon and ainyeles under METHODOLOGY). These processes play an important role in the diet of the people of Uganda, considering the recipes for the beverages.

Two basic processes are used. The first involves use of millet (Leusine coropana), sorghum (Sorghum bicolor) or beer sorghum, and/or cassava (Manihot esculenta) and water; the second uses "beer" bananas (Musa cultivars cvs), sorghum and water.

The first process involves germinating (malting) of the grain (millet, sorghum) which produces enzymes (amylases) that transform a portion of the starch to sugars (glucose and maltose). The sugars are then fermented by the yeasts such as Saccharomyces cerevisiae, Schizosaccharomyces pombe to ethanol.

The yeasts also grow and synthesize amino acids (glycine, leucine, histidine, lysine, aspartic acid and glutamic acid and vitamins) van Pee and Swings (1971). It is, therefore, essential that the products of fermentation not be clarified or filtered. Starch digestion occurs involving starch digesting moulds such as species of Mucor, Rhizopus, Amylomyces.

The combination of moulds and yeasts results in hydrolysis of starch to sugars which are then fermented. A lot of starch and proteins remain undigested and act as dietary components. The brews prepared here include:

Kwete, Amarwa/Ajon and Muramba.

The second process involves use of ripe beer banana and red sorghum varieties. Varieties of bananas are in many ways similar to cooking bananas but they are almost invariably used solely for beer (tonto) making.

Many indigenous fermentation products, either in form of beer (tonto, kwete, amarwa/ajon, muramba etc) or in form of bread or pastes, provide essential food values to the consumers in form of proteins, amino acids, vitamins and calories.

However, as Prof. Hesseltine (1965) has observed, the preparation of foods by fermentation with yeasts has certain disadvantages. Any additional processing results in additional time, equipment, and labour costs. There is loss of nutrients during washing in preparation for fermentation. The yeasts used in fermenting the substrate will require energy for growth. About 5% of the substrate will be metabolized. Under no circumstances is there any increase in substrate, apart from biomass yield of yeast cells. The question then arises as to why the fermentation of foods is used on such wide scale in Uganda and other parts of the world, where food is in short supply?

Millet and sorghum, together with cassava and beer bananas, because of drought resistance, are the crops par excellence for dry regions and areas with unreliable rainfall. These crops in their natural form are not very palatable. During the processes of steeping, malting/germinating and other processes, detoxication of bitter cassava takes place; in preparation of the grains and bananas certain undesirable odors and flavours are either destroyed or masked. A factor which cannot be underestimated is the monotonous diet of much of the world where protein, in the form of meat and dairy products is not available. Their foods lack flavour. Usually materials of plant, animal, mineral (inorganic), or synthetic origin are added to foods to make them more attractive or more palatable and appealing to the taste. Many people in the developing world because of their poor economies cannot afford appetizers. Fermentation almost invariably adds to the flavour of the food.

There is a lot that can be done to improve on quality and quantity of production of local brew, bread and pastes.

The processes of preparation and fermentation were observed to be carried out under extremely poor hygienic conditions, in villages visited during the study.

The beer, bread and pastes generally contain unpleasant tastes produced by contaminants such as Acetobacter and Lactobacter species which ferment ethanol to acetic acid and lactic acid. The general Hansenula and Kluyveromyces produce esters which are principal causes of spoilage of local foods and beverages. After 72 hours of fermentation the beverages and foods are completely spoilt and unpalatable. There is no technology or any effort put in place for pasteurization and storing the products of fermentation.

There is plenty of potential associated with modern breweries sugar refineries, pineapple and passion fruit juice canning.

Single-cell-protein production (S.C.P.) refers to the cells of yeast grown for their protein content. Cells of these yeasts, especially species of Candida, contain fats, vitamins and mineral salts. S.C.P. products are used either for animal feed or human food. They are potentially very important sources of amino acids, proteins, vitamins and mineral salts that can be prepared from otherwise inedible or unpalatable or low-quality waste materials.

For raw materials, carbohydrates such as sugars, or sugar-containing by-products, wastes and starches are available in Uganda in quantities required for an economically viable scale of production.

A portion of the crude sugar effluents and crude molasses and bagasse from sugar cane operation could be diverted to S.C.P production.

Pure strains of yeasts from fruits, grains and beverages in aerobic and anaerobic fermentation of sugars have been investigated. These could be further tried on crude sugar effluents and crude molasses and bagasse from sugar cane operations at Kakira and Kawolo sugar works; the sugar-containing effluents from pineapple and passionfruit canning factory at Kyabakuza near Masaka Town. These materials provide high potential for alcohol, CO₂ and SC.P biomass yields.

Uganda has breweries that produce residues of reasonably uniform composition throughout the year, at Nile Breweries at Jinja and Bell Beer Breweries at Port Bell. Recovery of brewer's yeasts leaves a liquid waste and after hydrolysis to simple sugars can be used as a potential carbon and energy source for S.C.P. production.

Coffee wet-processing or pulping in Bugishu yields effluents which contain a lot of soluble carbohydrate. This again could provide nutrients for S.C.P. production.

There is a paucity of written literature on yeasts in Uganda and a great demand for yeasts notwithstanding. Uganda Breweries, distilleries and Bread Industries (Hot Loaf, Tiptop etc..) depend on imported yeasts.

The local beverages brewers, beer brewers and bread-makers could save a lot of precious foreign exchange if the selected yeast strains were utilized.

For human food application the use of yeasts is limited to yeasts such as Saccharomyces cerevisiae and Candida utilis, that are accepted by regulatory and public health authorities as safe for human food use. Besides, if these organisms are to be consumed as a significant portion of the protein in the diet, they must be processed further to reduce nucleic acid content to below the levels that could lead to kidney stone formation or gout.

During this study, feasibility of using pure strains of yeasts from tonto in aerobic and anaerobic fermentation of tonto has been investigated. During fermentation, ethanol and biomass yields pH changes, flocculation ability and cell viability were investigated and changes in the cell morphology were also studied microscopically.

CHAPTER 2

REVIEW OF LITERATURE ON YEASTS: YEAST TAXONOMY AND APPLICATION OF YEASTS IN INDUSTRY

HISTORICAL PERSPECTIVE: The story of the yeasts.

The elucidation of glycolysis (Greek words: glycos=sugar, sweet; lysis = dissolution) and fermentation has a rich history. Indeed, the development of biochemistry and the delineation of these central pathways went hand-in-hand. Beer and wine production and bread-making by yeasts, the processing of milk to dairy products by lactic acid bacteria and the formation of vinegar by acetic acid bacteria show that microbes are among the oldest demonstrated organisms, Hans (1986). Fermentation is an ATP CO_2 - and ethanol-generating process in which organic compounds (most sugars) act as both donors and acceptors of electrons.

Fermentation can occur in the absence of O_2 . It was discovered (so has the story) by Pasteur who described fermentation as "la vie sans l' air" (life without air).

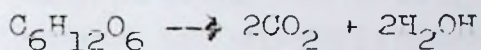
However, a key discovery is that one recorded by Hans Buchner and Edward Buchner (1897) quite by accident. They were interested in manufacturing cell-free extracts of yeast for possible therapeutic use. These extracts had to be preserved without using antiseptics such as phenol, and so they decided to try sucrose, a commonly used preservative. Sucrose was rapidly fermented into alcohol by the yeast extract (juice).

The next contribution was made by Arthur Harden and William Young in 1905. They added yeast juice to a solution of glucose and found that fermentation started almost immediately. However, the rate of fermentation soon decreased markedly unless inorganic phosphate was added. Furthermore inorganic phosphate disappeared in the course of fermentation, and so they inferred that inorganic phosphate was incorporated into a sugar phosphate. They also discovered that yeast juice contains two kinds of substances necessary for fermentation: "zymase" and "co-zymase". They found that yeast juice lost its activity if it was dialyzed or heated to 50°C . However, the inactive dialyzed juice became active when it was mixed with inactive heated juice. Thus activity depends on the presence of two kinds of substances, heat-labile,

nondialyzable component called zymaze and a heat-stable dialyzable fraction known as co-zymamaze. We know now that zymaze consists of a number of enzymes, whereas "co-zymaze consists of metal ions, adenosine triphosphate (ATP), ADP, coenzymes such as nicotinamide adenine dinucleotide (NAD^+).

During fermentation of carbohydrates by yeasts ethanol is the most important product. No oxygen takes part in the fermentation process. Yeasts are facultative anaerobes and can grow in the absence or presence of O_2 . In these facultative anaerobes, oxygen suppresses fermentation in favour of respiration, the Pasteur Effect, Loynen et al (1958).

Yeasts have been a favourite experimental subject in the research on basic metabolic pathways. The conversion of glucose to ethanol was formulated in 1815 by Gay-Lussac in its present form:



History of the elucidation of yeast fermentation is marked by disputes over whether alcohol fermentation from sugar is due to chemical contact catalysis or requires the activities of living organisms. The dispute seems strange today. The argument about the causation of fermentation was finally settled by L. Pasteur. Pasteur also showed that yeast can produce 20 times more biomass (cell materials) from a given amount of sugar under aerobic conditions than under anaerobiosis. The demonstration that in the presence of oxygen, fermentation is inhibited, the Pasteur effect, has become a model example of metabolic regulation.

CURRENT PERSPECTIVES: Introduction, Isolation, Identification, Taxonomy of Yeasts.

Yeasts serve many purposes. The selection of the most appropriate strains, species, and varieties for special purposes from the multiplicity available has been going on for a long time, Hans(1986) Weakly respiring, largely fermentative yeasts, so called "bottom yeasts" are used in the brewing of beer (Bell Beer, Nile Special, Pilsner, etc...)

The yeasts used for carbon dioxide and ethanol production, wine making as well as baker's yeasts are largely "top yeasts".

Baker's yeast (Saccharomyces cerevisiae) is used to produce carbon dioxide to "raise" the dough and it must, therefore, be a good fermenter. It is grown in tanks with good aeration. Ethanol is always produced as a by-product, but the relative yields of yeast cells (biomass) to ethanol can be varied by variation in aeration and glucose concentration.

Yeasts, are, therefore, commonly known as fermentative organisms because of their connection with brewing and baking. Brewer's and Baker's yeasts are strongly fermentative species of Saccharomyces and Kloeckera.

Some yeasts are entirely aerobic and lack the genes to cause alcoholic fermentation. These are usually grown for biomass yield: Rhodotolula, Cryptococcus, some species of Candida and (Torulopsis).

Other yeasts are strongly oxidative and weakly fermentative: Debaryomyces and Pichia, are strongly fermentative but, relatively, with weak oxidative activity, e.g. Saccharomyces carlsbergensis (the larger brewer's yeast).

Kluyver (1953) and Phaff et al (1966) made the following observations with respect to fermentation ability of yeasts:

- (i) Yeast which does not ferment D-glucose will not ferment any other sugar;
- (ii) Yeast which ferments D-glucose will also ferment D-fructose and D-mannose but not necessarily D-galactose;
- (iii) Yeast which ferments maltose will usually ferment lactose, eg. Brettanomyces claussenii.

ISOLATION

Fermenting beverages, such as tonto, kwete, amarwa/ajon and muramba can be considered as natural habitats of yeasts. Brewing in Uganda is a continuous process.

Aromatic odours of ripe fruits attract swarms of fruit flies and other types of insects. These transport yeasts from fruit to fruit. Many of these insects, especially Drosophila are closely associated with the process of brewing of tonto, kwete, amarwa/ajon and muramba. Therefore, these materials (fruits, grains and fermenting beverages) are good sources of yeasts.

IDENTIFICATION AND CLASSIFICATION - TAXONOMY OF YEASTS

Yeast identification and classification are vital both in Applied Microbiology and Biotechnology. As a study, yeast identification and classification was first established by Reess (1870) on pure cultures and brought to light the fact that yeasts are generally fermentative of carbohydrates. This led to the modern practice of employing physiological characteristics for identifying yeast species by Stelling-Dekker (1931) and Lodder (1952).

Guilliermond (1940) based his studies on sexual reproduction and suggested the phylogeny of the ascomogenous yeasts. Winge and Roberts (1958) carried out studies on yeast genetics and life cycles and laid down the modern concept of yeast sexuality and physiology as subsequently expounded by Wickerham (1955).

Alexopoulos (1979) gives the following description: The term yeast refers to morphological forms of Ascomycetes (perfect and imperfect) that have predominantly unicellular thallus, which reproduce a sexually by budding or transverse division (fission) or both, and that produce ascospores in a free ascus, originating either from a zygote or parthenogenetically from a single somatic cell.

The soma varies from family to family, and even within the same family. Some species are unicellular throughout their life cycles except for the asci that usually, but not always, contain more than one ascospore. Some yeasts produce a number of buds in succession that remain joined for a period of time forming pseudomycelium. Asexual reproduction is by fission, budding and formation of arthrospores.

Cell morphology:

Yeast cells vary in shape with species and even within the same species; with age and with the type of nutrient medium. The cells may be globose, ovoid, elongate, or rectangular in shape.

Colony characteristics useful in the taxonomy of yeasts include:-

- a) In liquid medium: sedimentation or flocculation; formation of ring islet; creeping up test-tube wrinkle formation.
- b) On solid medium: the culture may be mucoid, butyrous or butter-like, friable, coherent, matted, rough or smooth, glistening surface; flat or raised; pigmented (cream, yellow, red, pink, white).

Physiological characteristics:

Physiological characteristics are very important in determining yeast species. Lodder (1970), Barnett and Parkhurst (1974).

Van der Walt (1984) remarks that because the definition or description of the yeasts is neither clear-cut nor precise, yeasts are identified by recognition of overall similarities and affinities rather than of consistent and definitive characteristics. The author continues to say in absolute terms that no yeast can be identified or classified with confidence until a systematic study has been made of its cultural, morphological and physiological characteristics. "Let us call a yeast a yeast" says Flegel (1977).

Therefore, there are four distinct approaches to the problem of yeast identification and classification (taxonomy): The study of cultural characteristics, morphological, and physiological characteristics.

Identification involves the comparison of an "unknown" object (e.g. a newly isolated yeast) with all similar objects that are already known. If the "unknown" object matches up with a "known" we say that the former has been IDENTIFIED: if not it may be considered to be a "new" species, variety, or strain and, when adequately described, is added to the list of "known" objects. In practice this act of comparison is normally carried out not between two actual objects but between the "unknown" isolate and a recorded description of previously discovered yeast.

The identification of a particular yeast is made by matching the observed cultural, morphological and physiological characteristics with those recorded in standard monographs such as those of van der Walt and Johanned (1984) for yeasts previously described. Nomenclature is either a matter of a number in the yeast monograph or of a name deriving from its membership to yeasts suggested by phylogeny. The classification of yeasts is based upon grouping together yeasts of similar cultural, morphological and physiological characteristics (e.g. pellicle, rough, smooth, yellow, red, pink, pseudomycelium, budding, fission, globose; ferments glucose, fructose; assimilates salicin, arbutin etc.,). This sort of classification results in an arrangement in which yeasts of the same evolutionary trend are grouped together.

The aims of classification:

Why do we classify yeasts ?

Aims may be expediency or pragmatic. Since yeasts play an important role in industry, it is to have a system of classification which is practical and convenient.

The main purpose is to isolate, identify and name these organisms. Yeasts in each group have in common some cultural, morphological and physiological characteristics and which one can conveniently identify and catalogue among the YEASTS.

The first criterion in judging the validity of the characters used in the taxonomy of yeasts is their stability under a variety of environmental conditions. The character that changes "with the wind" is not a good one to use in separating taxonomic categories, Alexopoulos (1979).

A considerable amount of experimental work has shown that many characteristics used in delimiting genera of yeasts are quite unstable under different environmental conditions (such as pH, nutrients, aeration, etc...) in artificial media.

For the present study, however, effort was made to keep the yeast in their "natural" environment, the beverages (tonto, kwete, ajon) and examining their morphological characteristics.

Now the influence of evolutionary criteria on taxonomy during the post-Darwinian period that production of evolutionary relations between organisms, the phyletic classification, is often thought to be the sole aim of the taxonomist. It is necessary to consider whether other possible aims are valid, and, indeed, whether any other approach might lead to classifications of greater value than the purely phyletic, Hawker and Linton' (1988).

To do so we must first make the distinction between SPECIAL (or artificial or pragmatic) classifications and NATURAL classifications. A special classification is one made for a single, defined purpose: it assists in finding the answer to a specific question. A well known example is the classification of fermenters (yeasts that have a high ethanol yield) and assimilators (yeasts that yield high biomass).

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The purpose of this classification is to group together yeasts which can be selected for alcoholic fermentation or CO_2 yield and those for biomass production, for sale to brewers or bakers or for animal feed or pharmaceuticals, and to separate these yeasts from those other yeasts which do not have this significance. When an yeast isolated is identified as falling within a particular group of this system an answer to the question of a possible fermenter or ethanol producer or CO_2 producer, is obtained.

A further example is the system of classification used by medical mycologists, which places great weight on the pathogenicity of an yeast in separating it from otherwise very similar yeasts e.g. Candida albicans from candida-like Cryptococcales like Torulopsis, Torula, Rhodotorula etc.. The question answered here is whether a fresh isolate is likely to cause disease - a question of paramount importance to the medical mycologist.

Such classifications are perfectly valid and perform an important function, but they make no pretence to be natural systems.

What then constitutes a natural classification of yeasts?

Here our only lighthouse is the International Botanical Rules of Nomenclature deriving from Linnean analyses. The basic notion is that the organisms should be classified according to their essential nature or sensus stricto.... i.e. according to WHAT THEY REALLY ARE. Hawker and Linton (ibid).

When one attempts to apply the principle of sensu stricto to the classification of yeasts the taxonomist is faced with two connected difficulties. The first and most fundamental of these is that Aristotle's principle is one of deductive logic and yet a yeast taxonomist tries to apply it to situations where only INDUCTIVE logic is possible.

We cannot deduce that yeasts differ from other fungi. We can only recognise that they differ on the basis of our observations (because we do not know the essential nature of "yeast" or "fungus").

The second difficulty is that of yeast biological variation which makes the decision of which attributes are more "essential" than others even more likely to be arbitrary. Is, for instance, growth in vitamin free medium more essential than glucose fermentation; of growth in a medium enriched with NO_3 ?

After the publication of Darwin's works on the ORIGIN OF SPECIES, the earlier approach to classification was replaced by one that was thought to be at least equally "natural", namely, the phyletic system, Hawker and Linton (1988). It seems reasonable to argue that yeasts of similar "essential nature" would have shared common lines of descent. The great advantage to yeast taxonomists of the phyletic approach is that speculation about which attribute reflected most accurately the essential natures of yeasts may be replaced by decisions based on more tangible evidence of both phenotypical and genetic variation. The problem here would be deciding the taxonomic levels on which yeasts would be separated from each other. The homology of cultural and morphological characters may be doubtful. The doubt is a consequence of lack of evolutionary evidence. This refers to the identity of characteristics which appear to be similar but which may in fact have evolved along quite different lines of descent (convergent evolution), e.g. the similar structures of the Cryptococcales may be due to quite different processes and vice versa. Odds et al (1990) assert that numerical taxonomy analysis of imperfect yeasts of species in Candida and Torulopsis shows no basis for genetic separation.

Evidence upon which decisions on phylogeny are based include fossil records, biological variation (both phenotypic and genetic) and homology of various structures. If we consider yeasts with regard to these three pieces of evidence we find that:-

1. fossil evidence is virtually non-existent;
2. biological variation is great owing both to the ease with which mutations may be expressed (especially on artificial media in laboratory environment) and to extreme phenotypic adaptability e.g. the possession of inducible enzymes and other "switchable" control systems typical of micro-organisms,
3. the homology of attributes is frequently unknown. For example, we can divide all yeasts into perfect and imperfect yeasts; but there are some mechanisms by which yeast cells avoid sexual reproduction e.g. yeast infertility.

The lack of fossil evidence makes it difficult, if not impossible, to decide whether apparently similar yeasts have evolved from a common ancestral organism or whether convergence (or divergence) due perhaps to the selective pressures of sharing a similar habitat (tonto, kwete or ajon) has been responsible.

The following line of argument is developed by Hedges (1988). For the sake of illustration let us suppose that we have two yeast strains that share a large number of what appears to be similar characteristics (cultural, morphological and physiological). Let us further suppose that we also know that the lines of evolution of these strains converged from very different origins. Would the objective of a natural classification be best achieved by grouping these strains together on the basis of their natural overall similarity (phenetic classification) or by separating them so as to reflect their different origins (phyletic classification)? An argument for the phyletic approach might be that this best reflects the "essential natures" of the two yeast strains, to which the counter argument might be that, because of convergent evolution, their "essential natures" have become similar.

There is another way of approach on the aims of classification, by asking "what is the practical value of a natural classification on yeasts?"

The answer to this question is that, to be of maximum use, a natural classification should have good predictive value (information content). This means that if we are told that a yeast is classified in group X of a hypothetical taxonomic scheme, then from our knowledge of the general properties of group X we should be able to predict that the organism in question certainly has features a,b,c,d; probably has also x,y, and z. When approached from this view point it is possible to describe a natural classification as one which gives the greatest information to the scholar e.g. a mycologist interested in the classic taxonomy of yeasts.

In contrast, a special or artificial classification yields particular information to the specialized user, such as a beer brewer, baker, wine maker, a distiller, a pharmacist or a medical mycologist.

If we accept this distinction we may now resolve the hypothetical question:

What is the practical value of a natural classification?

It is clear that the phenetic classification would allow most general predictive properties, whereas the phyletic system would offer information that is primarily of use to the student of evolution.

It is now possible to see a connection between the grouping of organisms on the basis of phenetic classification and the use of statistical parameters in characterizing sets of data. For example, if we are told that a certain set of data is normally distributed about a mean value of 22 with a standard deviation of 0.3, we may predict that in roughly 95% of cases a single datum randomly selected from the set, would have a value between 21.7 and 22.3, or 22 ± 0.3 , although we should be unable to predict the exact value of the datum. This is similar to saying that organisms placed in hypothetical group X of a phenetic classification would be expected to vary about an "average" or modal form only between defined limits, although we should be unable to predict the exact complete description of a single yeast strain selected randomly from group X.

In the systems called NUMERICAL TAXONOMY it is possible to describe these limits in numerical terms. Again, if the range of yeast variation were so great that between each "typical" or modal strain there was an almost continuous gradation of "intermediate" strain (expected of natural variation) a phenetic classification would still have practical use in much the same way that a histogram may allow us to group and so handle what is in fact a continuous spectrum of data.

It follows, therefore, that the best phenetic classification is one built on comparisons based upon as many attributes as possible. Organisms which share a large number of attributes would cluster together to form a "natural" group and such groups would separate from each other at "points of rarity" i.e. at combinations of attributes which never, or very rarely occur. A phenetic classification based on the frequency of shared characteristics (overall similarity) is termed polythetic.

A phenetic classification based on the frequency of characteristics that uniquely describes a given object or an yeast is called monothetic classification. This principle is used in the construction of artificial dichotomous keys for identification of both higher organisms and micro-organisms. The essence of such a system is that certain key characters are selected, the possession of which automatically places the organisms to be identified into a group of organisms that uniquely possess these characters.

We see immediately that once a key character is selected it assumes great weight in determining the classificatory position of an unknown organism and we should therefore inquire whether we are justified in giving some characters more weight than others. It is obvious that, in principle, the use of key (catalogue) characters could nullify the aims of "natural" classification outlined above.

For example, if a new strain of yeast were discovered that differs in a single catalogue (key character for those already classified together in a group and yet a large number of characters is common with that group, we should be forced to place the strain in a separate group according to the monothetic system, whereas it would obviously join the existing group in a polythetic system.

In artificial classification weighting of certain characters would be useful, since they reflect the very criteria that were used in setting up the classification. For example, a special classification based on the criterion of fermentation would justifiably separate Saccharomycetales, which are perfect Ascomycetes from the largely imperfect Cryptococcales; or Candida utilis from Candida albicans (a pathogen causing human disease of the central nervous system), on the sole key character, pathogenicity. C. utilis is a fodder yeast. Candida also represents the sexual phases of various genera of ascomycetous and basidiomycetous yeasts, Kreger van Rij (1973).

It would appear logical to give more weight to those characteristics that are strongly correlated with others, a single one of these could then be used as a key character. Kluyver (1953) and Phaff et al (1966) made the following observations with respect to fermentation ability of yeasts:

- a) that a yeast which does not ferment D-glucose, will not ferment any other sugar.
- b) that a yeast which ferments D-glucose will also ferment D-fructose and D-mannose but not necessarily D-galactose.
- c) that a yeast which ferments maltose, will usually ferment lactose e.g. Brettanomyces clausenii.

Yeasts which are able to ferment polysaccharides, such as inulin and starch are rare. Saccharomyces fragilis is an example of a yeast which ferments inulin well, and Endomycopsis fibuligera ferments starch fairly well, Phaff et al (1966).

Production of amyloid compounds is characteristically associated with Cryptococcus. The mutual sexual agglutination of Hansenula; its utilization of NO_3 ; is pigmented red. These characteristics place Hansenula into a distinct group; growth by binary fission in Schizosaccharomyces; growth in 20 ppm crystal violet by Saccharomyces delbrueckii, etc...

Two things follow from these examples. First, that the same weighting would be obtained by giving equal weight to each of the individual correlated characters, which would then act in concert in influencing the classificatory position. Second, that if we eventually found that all of these correlated characters originated from a single genetical feature then their weight would paradoxically disappear since all would be expression of the same thing, Sneath (1957a). We are, however, in doubt about the homology of apparently similar characters in yeasts, nor do we at present know the precise genetical reasons for observed correlations between characters, Hedges (1988). In this position of ignorance, the best natural classification is one based upon comparison of yeasts with respect to as many characters as possible, each character being given equal weight in contributing to the grouping and separation of different organisms (i.e. a polythetic system), Sneath et al (1973). For example the usual variables for classification of yeasts include: cultural tests(14); morphological tests (15); physiological tests (42), a total of 71 characteristics. These are the standard characteristics given by Kreger van Rij et al (1973). These form the basis for the conventional methods of yeast identification and taxonomy.

Once such a classification has been made it is then possible to search for key characters which may be used in a method of identification. Single key characters as those used in dichotomous system were not practical.

Rather, a set of the 58 or so characters were examined together in order to narrow down the possible classificatory location of an unknown yeast strain.

The idea of phenetic classification is based on characters of equal weight and it stems from the eighteenth century French botanist, Michel Adanson, and now we can apply the term Adansonian to phenetic classification.

It follows, therefore, that in this study we shall use two systems of classification of yeasts: The conventional system by Kreger van Rij et al (ibid) and Adansonian system or Numerical taxonomy.

The most widely used general classification of yeast is that of Kreger van Rij (1984); Lodder (1970); Van der Walt et al (1984); Phaff et al (1987);

The system is a mixture of phenetic classification and a quasi-evolutionary approach.

CLASSIFICATION: Classification is the subject of taxonomy. Classification means the ordering of groups of yeasts into higher units. This is done in several stages. The basic unit, that is, the pure culture of the isolated yeast is the strain, named say, $MR_{ij}Y12$, $MRU_{ij}Y10$, $JTY16$, etc... The strains are collected into the species, say $MR_{ij}Candida Y16$, $MRU_{ij}Saccharomyces Y 64$. The classification requires adequate description of strains and proceeds by comparing the properties of various groups. As mentioned before, two kinds of classification were used: Conventional classification, which is both pragmatic or artificial and phylogenetic, and numerical taxonomy.

Classification in the current study means the act of arranging a number of yeasts into groups in relation to characteristics (cultural, morphological or physiological) of these yeasts for various applications.

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Taxonomy (Greek, taxis = arrangement, nomos = law) is concerned, inter alia with definition of the AIMS of classification, the DESIGN of rules by which arrangements may be achieved, and with the EVALUATION of the end results. In short, TAXONOMY may be described as the scientific study of classification.

A pragmatic or artificial classification of yeasts is less demanding than phylogenetic or numerical taxonomy. It aims to group yeasts according to their similarities so that they can be recognized and identified, e.g. fermentative and non-fermentative yeasts; brewer's and baker's yeasts; aerobic and anaerobic yeasts; etc... Such a system is designed for use as a determinative key. The key or catalogue of characteristics can be used for classification of new isolates.

CONVENTIONAL YEAST TAXONOMY

Cultural characteristics:

- a) Growth in liquid media: Observations are made on malt extract or 2% glucose-yeast extract broth. The species may exhibit sedimentation (flocculation) formation of a ring islet; formation of dull wrinkled surface; growth may be creeping; moist or mucoid and glistening.
- b) Growth on solid media: e.g. on malt agar; yeast extract-malt extract agar; nutrient agar-glucose; yeast growth may be mucoid; butyrous (butter-like) friable; coherent; matted; margin smooth or dented. In genera Lipomyces and Cryptococcus matted or coherence is generally associated with the production of pseudomycelium, or true mycelium. The species may exist either as rough (R) and smooth (S) or mat (M) and glistening (G) forms on solid media. In Hansenula there are G and M forms. The G form species produce appreciably less ethanol.

Growth may be strongly pigmented by the production of certain pigment types.

The presence of creamy, yellow, or orange, and red carotenoid pigments is highly characteristic of the genera Rhodotorula, and Sporobolomyces. In Beijerinck (1918), van der Walt (1952), Kluyver et al (1953); Cook and Slater (1956) report the formation of red pigment in the yeast Candida pulcherrima.

Yellow, orange and red in Rhodotorula; bordeau red in Sporobolomyces; red in Metschnikowia. Other yeast colonies have white to cream or lighter shades of brown.

The colony may be fast or slow spreading on surface of the medium; colony may be flat or raised; may produce esterical or aromatic odour etc...

Cultural characters provide 23 units for conventional taxonomy. If the character is present it is given a(+), if absent a(-).

Morphology (microscopic features of cells): these include characteristics of vegetative reproduction. Vegetative or asexual reproduction (in morphology broth or morphology agar) occurs in yeasts by fission, budding or by a combination of these two processes, in liquid medium.

Reproduction by fission is a typical characteristic of the Endomycetaceae and Schizomycetaceae.

Reproduction by budding is typical characteristic of the largest group of the yeasts, the saccharomycetaceae the Saccharomyces type. These are: Saccharomyces, Endomycopsis, Fabospora, Kluyveromyces, Schwanniomyces, Debaryomyces, Hanseniospora, Saccharomycopsis, Citeromyces, Pichia, Hansenula, Pachysolen, Dekkera, Saccharomycoides, Wekerhamia and Nadsonia.

Asexual reproduction by budding is by far the commonest and the classical form of reproduction in yeasts, Nickerson (1963).

The concomitant occurrence of budding and fission may be observed in a number of genera e.g. Endomycopsis, Candida and Trichosporon.

Characteristics of vegetative cells:

- a) The morphology of the vegetative cells grown in liquid and solid media: The shape of the vegetative cells is useful as a taxonomic criterion, since their form is closely connected with the way in which they are produced. The cells may be spheroidal, subglobose, globose, ovoidal, ellipsoidal, cylindrical, or fusoid. In many cases the cell shape is so characteristic that it may be employed in generic differentiation, Lodder et al (1971); Abalaka and Opera (1990) for example described 24 cultures as follows:

<u>Culture No</u>	<u>Microscopic(morphological) features of cells</u>
1.	Mostly budding, oval, many clusters
2.	oval, round and single, mostly budding
3.	round cells, some budding, few in clusters
4.	single, oval and round, mostly budding
5.	oval and elongated, mostly in singles except when budding
6.	singles, mostly budding
7.	singles, mostly budding, branched
8.	oval, mostly budding clusters of 2-3, cells
9.	oval and round, few budding
10.	actively budding, oval, mostly singles or in buds
11.	oval and singles, mostly budding
12.	mostly singles, budding
13.	mostly singles, oval and round
14.	actively budding, oval, mostly singles or with buds
15.	singles and flocculates, budding cells
16.	round and oval, mostly budding
17.	singles and flocculates, many budding
18.	round and oval, single, flocculates; budding
19.	round and oval, about 50% singles, budding
20.	similar to No 19
21.	large, oval, flocculates well, few budding

- 22. large, oval, many budding
- 23. similar to No 22
- 24. round, mostly singles, few budding

Modes of budding are: multilateral or bipolar.

b) Formation of pseudomycelium: If a yeast reproduces vegetatively by budding the mature bud may either detach itself immediately or may adhere to the mother cell and eventually give rise to clusters or chains in aggregates may result in formation of pseudomycelium or pseudohyphae. Lodder and Kreger van Rij (1952) differentiated between Torulopsis and Candida merely on the presence of pseudomycelium in Candida.

The pseudomycelium useful in differentiating the genera of the family Cryptococcales (asporogenous yeasts). The genera include:- Cryptococcus, Pityrosporum, Kloeckera, Brettanomyces, Candida, Rhodotorula, Torulopsis and Rhodospiridium.

For the study of pseudomycelium formation, maize agar, potato glucose agar, or morphology agar are normally used.

Physiological characteristics

Introduction

There are differences in metabolic patterns, substrates which can be utilized and the end products produced by yeasts.

Yeasts are microorganisms. The substances from which microorganisms synthesize their cell materials and obtain their energy, the nutrients, are dissolved in water. Different yeasts have very different requirements for composition of nutrient media as well as for other environmental conditions, e.g. temperature, pH, osmotic pressure, etc... Because of this, numerous recipes for the composition of yeast media have been devised in studying physiological properties of yeasts,

Nutrient media must fulfil the following minimal requirements: they must supply as utilizable compounds all elements that take part in the synthesis of the cell substances. These include a variety of mineral salts, growth factors (vitamins) and amino acids or modified proteins, e.g. peptone. Yeasts obtain their cell carbon and energy from organic compounds. These are mostly sugars, e.g. glucose (dextrose).

Yeasts are dependent on molecular oxygen (O_2). The main function of oxygen is to act as a terminal electron acceptor in aerobic respiration.

In their relation to oxygen, yeasts can be aerobic or anaerobic or both (of Pasteur Effect). Most yeasts are facultative anaerobes. They can grow either in the presence or absence of oxygen.

Although the number of species of yeasts which can ferment hexoses, disaccharides and trisaccharides is large, yeasts which are able to ferment polysaccharides, such as inulin and starch are relatively rare. Saccharomyces fragilis is an example of a yeast which ferments inulin well and Endomycopsis fibuligens ferments starch fairly well, Phaff et al (1966).

In general, the characteristics which have proved most useful in yeast taxonomy are those associated with the utilization of carbon and nitrogen sources; growth factors requirements; the fermentation of typical characteristic metabolites, such as esters, van der Walt et al (1984).

Utilization of carbon compounds

A yeast developing on a carbohydrate source must either be able to ferment it or to utilize it by oxidative respiration, van der Walt et al (1984).

a) Fermentative utilization of carbon compounds:

The ability or inability to ferment carbohydrates to ethanol and carbon dioxide is a most useful characteristic for differentiating species, occasionally useful in demarcating genera. For example, the genus Saccharomyces is characterized by the vigorous fermentation of one or more sugars while other genera e.g. Lipomyces and Rhodotorula are strictly nonfermentative, while in other genera, e.g. Hansenula has species varying from nonfermentative to strongly fermentative species.

A number of tests have been devised to determine the presence or absence of fermentation as indicated by the production of CO₂ under conditions which permit the fermentative utilization of the sugars. The tests may be conducted in a variety of specially designed vessels either of the open type, e.g. Durban and Einhorn tubes or of the closed plug tubes

Guerra, Langeron and Guerra (1938), the Winge fermentometer, Winge and Roberts (1950a), the syringe fermentatometer of Morris and Kirsop (1953) and the quantitative van Iterson-Kluyver fermentometer, Kluyver (1914). Durham tubes containing 2% sugar solution have been found to be the most useful. The fermentation rate may be classified as rapid (+++) medium (++) or slow (+) or absent (0) after 48 hours.

The range of carbohydrates employed in the fermentation tests include: monosaccharides; trisaccharides; pentoses; polysaccharides and their derivatives. Of these, glucose, galactose, sucrose, maltose, lactose, melibiose and raffinose have been found to have the greatest application.

b) Oxidative utilization of carbon compounds: oxidative respiration tests are far more sensitive than fermentation tests for detecting the presence of enzyme systems and, therefore, more useful in taxonomy, Barnett (1974).

Assimilatory (oxidative) characteristics such as the ability or inability to utilize a carbon compound oxidatively, are employed for describing species and genera, Lodder et al (1971).

Biochemists have found out that many of the chemical processes in cells occur in chains of reactions in which, for instance, chemical a changes to chemical b, then chemical b changes to chemical c and so on, Lehninger (1982), Bartnick-Carcia (1973), Winge and Roberts (1958), Bainbridge (1980), Old & Primrose (1985). Chemical reactions in living organisms are catalysed by enzymes. That is to say, in enzymes can increase or decrease the rate of a chemical reaction.

Each living organism resembles its ancestors in most of its characteristics, including metabolic characteristics. The maintenance of specific properties, that is, the constancy of characters over the generations, is called heredity.

These characters are transferred in form of genes. Metabolism is controlled by genes. This means that similar genes will have similar metabolic pathways, that is, they will be synthesizing the same enzymes.

The utilization of inositol is considered to be characteristic of Cryptococcus and inability to use lactose appears to be a feature of Saccharomyces. Some genera e.g. Cryptococcus and Debaryomyces are characterized by the utilization of a wide range of compounds while others e.g. Sacchromyces and Trigonopsis assimilate relatively few carbon compounds, Barnett and Kornberg (1960).

Tests are available used to determine the ability or inability of yeasts to utilize carbon compounds.

There are three main tests: In the first type of tests, the compound is added to a complete liquid basal medium in which the compound serves as the sole source of carbon.

As the test runs over several weeks, it permits the adaptation of the yeast to certain compounds. Blanks (controls) are used, consisting of the inoculated basal medium, devoid of any carbon source, Wickerham and Burton (1948).

Aeration or agitation gives rise to more rapid results Barnett and Ingram (1955) and Ahearn et al (1960).

The second type of test involves use of purified agar, first used by Saito (1922). The inoculum is applied either as a steak or dot. A control is used since agar often contains traces of assimilable compounds which may support growth.

The third test, by Beijerinck (1889), consists in employing a cooled, liquified basal agar medium devoid of any carbon source. The medium is seeded with the yeast inoculum. A pour plate is then prepared on which small amounts of the compounds to be tested are placed after solidification of the agar. The compounds selected for the tests include: glucose, galactose, sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinoses, melezitose, inulin, starch, L-xylose, D-arabinose, D-ribose, L-rhamnose, ethanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, salicin, DL-lactate, succinate, citrate, arbutin.

Utilization of nitrogen compounds

The ability to utilize nitrogen compounds is useful in yeast taxonomy. The utilization of potassium nitrate and sodium nitrate have proved to be very useful taxonomic characteristics, Ladder (1971).

For taxonomic purposes the ability to utilize a nitrogen source is assessed by determining growth under aerobic conditions.

The liquid assimilation test devised by Wickerham (1946, 1951) is used. In using the liquid test medium it is necessary to verify the assimilation of a specific compound by transferring a small inoculum from the first tube to a second, since, as a result of the excretion of assimilable nitrogen, yeast cells which are initially well nourished may proliferate considerably even when an external nitrogen source is absent, Nielsen (1936); Thorne (1945). The use of blanks (control) tube is therefore obligatory.

a) Utilization of nitrate:-

Many genera are characterized by their inability to utilize nitrates e.g. Saccharomyces, Kluyveromyces, Pichia, and Debaryomyces. Other genera e.g. Hansenula, Pachysolen and Citeromyces all utilize nitrate. Among the imperfect (asporogenous) genera both nitrate-positive and nitrate-negative species occur e.g. Candida and Torulopsis.

b) Utilization of nitrite

Certain species of the genera Debaryomyces e.g. Picha-pinus, utilize nitrite but not nitrate, Wickerham (1957); Kreger-van Rij (1964). In testing the assimilation of nitrite, toxic effects due to nitrous acid formation may occur in media with a pH below 6. Wickerham (1957) recommended the use of 0.26 g nitrite per 100 ml tenfold concentration medium.

Growth in vitamin-free medium.

Ability or inability to grow in a synthetic medium devoid of vitamin is a diagnostic criterion in yeast taxonomy, Wickerham (1951). Vitamins employed include biotin, calcium pantothenate, folic acid, inositol,

Brettanomyces, Kloeckera, Metschnikowia, Hanseniaspora, Dekkera and Kluyveromyces are unable to proliferate in the absence of an external vitamin source. Hanseniaspora and Kloeckera have been found to have an absolute requirement for inositol and pantothenic acid. Dekkera, Metschnikowia and Brettanomyces species require biotin and thiamine, Wickerham (1951), Wickerham and Burton (1962).

The detection of vitamin requirements is based upon cell proliferation under aerobic conditions in standardised, complete, synthetic media, devoid of growth factors, i.e. devoid of vitamins of the B-group. Yeast cells that are initially well nourished, may proliferate considerably in the absence of an external vitamin source because of a carry-over of previously acquired growth factors. Subculturing is continued in the vitamin-free medium until this internal source of growth factors is depleted.

Production of extracellular amyloid compounds

Aschner et al (1945) and Mager and Aschner (1947) studies the production of starch-like material by encapsulate yeasts. Polysaccharide in the capsule gives a blue colour with Lugol's iodine solution. The pH of the medium should be maintained below 5. Lodder and Kreger -van Rij (1952) applied this criterion to the genera of the Cryptococcales and reported that 'starch' production was manifested in all species of the genus Cryptococcus and in some species of Candida, Bullera and Trichospora, and absent in Rhodotorula, with the exception of one species. The formation of 'starch' was employed to separate the genera Torulopsis and Cryptococcus.

Pigment formation

Although the colonies of most species of yeast appear white or cream or yellowish or white with a light greyish or yellowish cast, carotenoid pigment are

produced by species of one genus of Sporobolomyces and two genera of asogenous yeast Rhodotorula and Cryptococcus species produce red and yellow pigments; Cryptococcus produce lighter yellowish or pinkish pigment. Candida streaks assume a deep marron-red colour. The pigment called pulcherrimin is produced by Candida Fabospora, Hansenula and Pichia and by some strains of Saccharomyces growing in biotin deficient media. The "black yeast" Pilularia usually forms colonies which are initially white. A few days after go through pinkish, greenish, deep brown, to jet black stages of coloration. In cases where the concentration of pigment is high, the bright colour is useful characteristic when considered in conjunction with other properties.

Ester production

Pichia and Hansenula are best known as contaminants of alcoholic liquors. They grow as dry, wrinkled pellicles on the surface of the fermenting liquid. If fermentation takes place, the main products are esters and not alcohol. The genus Brettanomyces and Kluyveromyces have been diagnosed by the characteristic aroma they produce. Ethyl acetate has been found to be the commonest and most readily detectable ester formed by yeasts. Ester odour has been employed for taxonomic purposes, Wickerham (1951), Wickerham and Burton (1951).

Selection of yeasts for controlled aerobic and anaerobic fermentation

The feasibility of using pure strains of yeasts from palm-wine in aerobic and anaerobic fermentation of molasses has been investigated by Abalaka and Ogora (1990). It was found that during fermentation, alcohol and biomass (oxidation) yields, pH changes, flocculation ability and cell viability were independent of the origin of the yeasts.

The yeasts selected had been isolated from different types of palm-wine obtained from different geographical locations. This confirmed the observation of Okafor (1972) that the distribution of yeasts did not seem to depend on the locality from which the juice was obtained or the type of palm. The study for tolerance of the toxic materials in the molasses revealed that only 7 of the 24 yeast types could survive long periods of storage in fermented molasses, a condition which is sometimes experienced in alcohol fermentation companies, especially in developing countries. These 7 types show more than 50% viability after 3 weeks in fermented molasses.

Most of the yeast types showed a high level of stability (preservation ability) after 4 weeks of storage on agar slants (nutrient Agar-Glucose = NAG). Flocculation is important for separation of yeast cells from the fermented molasses and a good yeast type should show some degree of flocculation, Abalaka and Opora (1990).

The yeasts gave rather low yields of biomass under both aerobic and anaerobic conditions when oxygen was a limiting factor during fermentation. Values obtained for both alcohol and biomass yields in pure anaerobic fermentation demonstrated that most of the yeasts fermented and produced high yields of ethanol but low yields of biomass. This showed that most of the sugar utilized by these yeasts was converted into ethanol.

Taking all these characteristics into consideration Abalaka and Opora (1990) concluded that the selected yeasts appeared to show good features and had been selected for further studies. The following fermentation characteristics would be sought:

i) high yield of ethanol and low yield of biomass under anaerobic conditions;

- ii) high yield of biomass under aerobic conditions for the purpose of cell propagation;
- iii) little change in pH during fermentation, i.e. low level of acid production;
- iv) ability to withstand toxic materials in fermented materials should the need arise to store the yeast under such conditions;
- v) effective flocculancy, to aid in yeast harvesting.

Ethanol tolerance

Yeasts vary in their susceptibility to ethanol concentrations. Ethanol is one of the products of the metabolism of glucose by yeasts. The amount produced is directly related to the concentration of glucose in the medium, Zeuthen et al (1988). Some yeasts produce ethanol but are relatively intolerant of ethanol in their environment. In Candida albicans 1% ethanol retarded growth and 5% ethanol completely suppressed it for 24 hours at 37 C. The ethanol tolerance of C. albicans was relatively low in comparison to some other species of yeasts. Casey and Ingledew (1986). The measure of inhibition was the length of the lag period of growth and the generation time during exponential growth, Zeuthen et al (1988) tested the effect of ethanol on yeast cell viability. A 90% reduction in the number of colony forming units (c.f.u) ml^{-1} occurred after 10 hours at 5% ethanol, by 7 hours at 7% ethanol, by 6 hours at 9% ethanol, by 3 hours at 11% ethanol and in as little as 1 hour at 13% ethanol. These characteristics are important in storage of fermenting beverages or prolonged maturity period of the beverages, e.g. wine, beer etc...

Production of ethanol from glucose

Flasks of YNB (Yeast Nitrogen Base) supplemented with different concentration of glucose were inoculated with cells of C. albicans.

Samples taken at regular interval were tested for ethanol production and glucose depletion. In 1% glucose strains produced 0.33% (w/v) ethanol after 17 hours incubation, when all glucose had been utilized. After a long period of about 4 days the ethanol began to disappear as a result of utilization by the yeast. In 2% of glucose the yeast produced twice as much ethanol in the same time and did not utilize the substance during the remaining incubation period. In 5% glucose about 5% times as much ethanol was produce as had been from 1% glucose but a longer period of time was required which might have reflected the toxicity of the end-products of the cells. In a similar way larger amounts of ethanol were produced over longer periods of time from starting concentrations of glucose of 7, 10, and 15%, Zeuthen et al (ibid).

Substantial amounts of ethanol can be produced from relatively large concentrations of glucose in the medium. The yields were 60 - 80% of those expected. Final concentrations of 0.3 - 3.0% ethanol did not reduce the viability of the cells but concentrations of 4% or greater sterilized the cultures even though fermentation continued for a time after cell death. Thus the fermentation rate was less sensitive to ethanol than was growth rate, D'Amore and Steward (1987).

Zeuthen et al (ibid) showed that exposure of yeast cells to 7% ethanol at 23 C strongly suppressed normal protein synthesis.

Tolerance of ethanol among microorganisms has been measured in a variety of ways, Casey and Ingledew (ibid), Juroszek et al (1987).

Casey and Ingledew (ibid) showed that alcohol-producing species of Saccharomyces had high level of alcohol tolerance. Zeuthen et al (ibid) observed that 11% ethanol reduced viability of C. albicans by 90% in 3 hours.

This same reduction in viability of a strain of Saccharomyces cerevisiae required nearly twice the concentration (21%) of ethanol. The basis of resistance is unknown, Juroske et al (ibid).

Thermal tolerance

Spittstoesser et al (1975) studied the influence of wine composition on thermal resistance of potential spoilage yeasts.

Pasteurization studies were conducted on 29 yeasts. In general yeasts were heat resistant in wine. Ethanol was the wine constituent that had the greatest effect on thermal resistance. D-values for all test species were inversely related to the ethanol concentration. The response of yeasts to other factors such as pH and sugar concentration varies with the species.

Bottled wines that have been sweetened or which contain some residual fermentable carbohydrates are susceptible to spoilage by yeasts and aceto-lactic acid bacteria, Amerine and Joslyn (1970), Rankine and Pilone (1973), Vaughn (1955). Multiplication of these organisms often results in off-flavour as well as objectionable turbidity and sediment, Amerine and Joslyn (1970). One of the methods employed to prevent spoilage is pasteurization. Few quantitative data are available regarding the conditions necessary to impart biological stability to wine. Many heat processes differing in severity are used. Some wineries use flash pasteurization at 80°C or higher. Others use hot bottling process in which the wine is filled into bottles at temperatures ranging from 45 to 70°C.

Most of the yeasts investigated yield D-values of under 0.5 minutes when heated at 49°C. The most heat-resistant yeast a strain of S. cerevisiae gave an average D-value in five experiments of 1.6 minutes,

Of the different wine constituents that were studied, ethanol again had the greatest effect on heat resistance. The resistance of both bacteria and yeasts was reduced in the presence of ethanol, Carrol and Lopez (1969), Jacob et al (1964), Schander (1959). Schander observed that differences in alcohol concentration of only 3% had a marked effect on survival of yeasts.

The effect of pH on heat resistance

Wines and local brews (tonto, kwete, etc) have pH ranging from 3 to 4 from pilot studies. Results show that individual species of yeasts respond directly to hydrogen ion concentration. Yeasts showed increased D-values as the pH was raised between pH 3.4 to 4.0, Splittstoesser et al (ibid).

Glucose is another common wine constituent to which individual species respond differently. Thus, the addition of 10% glucose to wine increased the D-value, affording protection to the yeasts. These results show that sweet wines require greater degree of pasteurization than dry wines (with less sugar).

Researches show that different gene products are implicated in sensing the nutritional and physical conditions of the media, Hanes et al (1986). Mutants of Saccharomyces cerevisiae had generation times similar to that of the parental strain when growing with glucose or ethanol as carbon source, and on sporulation medium in the absence of glucose they behave like the wild-type. In contrast, in the presence of glucose, the mutants raised the pH of the medium and utilized glycogen after an initial period of accumulation. Addition of glucose to sporulation medium suppressed sporulation, Salmon et al (1988). Mutants were able to sporulate on solid sporulation medium in the presence of 1% glucose, a concentration that completely inhibited sporulation of the wild-type strain.

Sporulation in the mutants was inhibited by 5% of glucose except in one mutant, which also sporulated (about 50% sporulation) in the presence of 1% glucose. In all cases the diploids did not sporulate in the presence of glucose, Salmon et al (1989). During sporulation, the pH of the medium increases because acetate ions are taken up and replaced by bicarbonate and carbonate ions in greater concentrations than the protons excreted, Powell (1967). In contrast, utilization of glucose in minimal medium is accompanied by a decrease of pH due to proton excretion of the plasma membrane ATPase, Serrano (1980). In the absence of glucose, the mutant in sporulation medium gave the same high final pH as the wild-type. However, in the presence of glucose, the pattern of pH change was completely different. The pH decrease observed with the parent strain was not observed with the mutant, Salmon et al (1989).

Being able to control growth rate of population of cells and fermentation at constant temperature is a very useful facility both for industrial processes and as an investigative tool for taxonomic studies on cell growth, division and fermentation, Eamon et al (1989).

Batch culture methods rely on altering the overall nutritional composition of the medium or by changing the carbon source. In many industrial processes such as brewing, distilling and wine making, yeast is grown in a medium which is rich in sugars. In these circumstances, growth is virtually anaerobic and the sugars assimilated are metabolized to CO₂ and ethanol fermentation. Even if the vessel is well aerated, the sugars are still metabolized to CO₂ and ethanol until the sugar concentration has been reduced to a very low level.

Continuous culture

When cells are grown in batch culture, growth only continues for a short time before nutrients become depleted and growth stops. Continuous culture is a technique developed to permit cells to be grown at a constant growth rate for long periods of time. It is achieved by adding nutrients continuously to the growth vessel and removing spent medium together with the cells suspended in it at the same rate. This ensures continuous yield of biomass.

Yeasts in industry

The value of alcoholic beverages produced has been assessed at $\text{€}2.4 \times 10^{10}$ per year!!, Berry (1988). The main property of yeasts which has made it a valuable organism to man is its ability to convert high concentrations of sugar into ethanol with high efficiency.

Fermentation process

a) Preparation of raw materials:-- in fruits and malted grains sugars present are readily assimilated by yeast. The fruits and malted grains (germinating millet, sorghum and maize) are crushed to release the sugars. In the production of coloured juice skins of fruits are left in contact with the juice throughout the fermentation. The final flavour of the beverage is dependent upon a variety of factors, e.g. the quality of the fruits used; addition of roasted and crushed grains make a major contribution to the flavour.

Starch cannot be assimilated by yeast. So the important part of the technology of brewing (in industrialized and not-so-industrialized third world) is the controlled germination of the grain to produce amylase enzymes. This process is known as malting. The malted grain is dried, roasted by "kilning" before being ground.

During the drying/roasting process coloured compounds develop by the carametization of the carbohydrates which are important in giving the correct colour and aroma to the beverage.

The malt is ground up and mixed with water in the mashing process. During this process the amylase enzymes (if not yet denatured break down most of the starch to metabolisable sugars such as maltose. In some tribes of Africa the "green malt" provides the active enzyme. Some of the starch, the dextrin fraction, however, is not broken down and it is not metabolizable by yeast. So it remains intact throughout the fermentation phase and gives body to the beverage. The extract prepared during mashing process, called wort, is mixed with green malt and is used as the fermentation medium for the beverage production. In beer-making the extract is filtered off from the debris of the grain before use.

b) The fermentation stage: The fermentation phase in the production of the alcoholic beverage is normally a single stage batch process (a closed system) which is carried out in containers. Some beverages are fermented by selected yeast strains to produce a beverage of the correct strength and flavour. Traditional beverages (e.g. tonto, kwete, amarwa, etc.) fermentation is carried out by the natural flora from the skins of the fruits and surfaces of grains and from swarms of flies that invade the brewing area. So a wide variety of species contribute to the fermentation, e.g. Brettanomyces, Candida, Debaryomyces, Hansenula, Sacchromyces, as well as Schizosaccharomyces, Berry (1988). After inoculation, the fermentation proceeds with a rapid growth phase which is associated with a rapid period of ethanol formation and sugar consumption, Berry (ibid). After this period, the rate of alcohol formation slows down.

The fermentation period may last a few hours (tonto, kwete, amarwa (interviews), 12-18 hours; a few days (beer), to several months or years (wines).

Maturation and distillation

Local brews no maturation period is possible because of the presence of spoilage microflora (yeasts and bacteria). In beer, lagers and some wines maturation may last from a week to many years. Many wines which are distilled in production of whisky and brandy, the beverages take several years maturing.

In beer production, the maturation phase is important for the removal of certain undesirable volatile compounds, and for the precipitation of polyphenolic compounds, which can give rise to turbidity in beer. In the maturation of wine (from bananas, grapes or palm juice) malic acid is removed from the wine by growth of bacteria in the so-called malo-lactic acid fermentation. This and other biochemical changes lead to an improved flavour in the wine during maturation Berry (1988).

Gasohol production

Gasohol is a mixture of ethanol and petrol. Theoretically, ethanol can be produced from any carbohydrate source which can be converted into a form which can be metabolized by yeast. To embark on gasohol production as an alternative source of energy the following must be considered. Although 1 kg of ethanol has a gross calorific value of 29.7 MJ, only a part of that is a net energy gain, since some energy has been put into the system during the growth of the crop (banana, sugar cane, maize, millet cassava, sorghum etc...), the transport of the crop to the distillery and the distillation process itself, Hessel tine (1965).

Baker's yeast and Biomass production

Baker's yeast is selected to obtain a strain which produces CO_2 at a high rate in dough. At the same time the baker's yeast selected can be used in biomass production. Since baker's yeast is added to baking flour at a concentration of 1% on a weight basis, it represents an important source of microbial biomass in human diet. The amino acid composition and vitamin content of baker's yeast is shown below, Berry (ibid).

Nutritional value of yeast

		wt^{-1}	
amino acid	(g 100g dry)	vitamin	($\mu\text{g g dry wt}^{-1}$)
lysine	3.2	thiamine	165
valine	5.5	riboflavin	100
leusine	7.9	niacin	585
isoleusine	5.5.	pyridoxine HCl	20
threonine	4.8	folic acid	20
methionine	4.8	calcium panthothenate	100
phenylalanine	4.5	biotin	0.6
tryptophan	1.2	paraaminobenzoic acid	160
cystine	1.6	choline chloride	2710
histidine	4.0	inositol	3,000
tyrosine	5.0		
arginine	5.0		

Comparison of diet with and without sorghum/maize
beer (Source: B.C. Platt (1964))

Amount of food eaten (g)

<u>Food item</u>	<u>Diet without beer</u>	<u>Diet with beer</u>
Grain, whole meal	350	137.5
maize, 60% extraction	350	137.5
grain beer	-	2840 ml
vegetables	130	130
sweet potatoes	470	470
kidney beans	30	30
vitamin B	0.002	0.00195
riboflavin	0.00113	0.00232
nicotinic acid	0.0203	
calories	3016	2979

The caloric content of the two diets is quite similar, only 37 calories being lost in the diet containing beer. The most notable improvement is the doubling of the riboflavin and the near doubling of nicotinic acid in the diet containing beer, because of synthesis of the vitamin during malting and fermentation. Pellagra, which is relatively common in people subsisting on maize, is never noted in those consuming usual amounts of sorghum/maize beer.

NUMERICAL TAXONOMY

Numerical taxonomy aims at a more objective system of classification. This started originally with adoption of the Adamson Principle that all properties (characteristics) for classification should be given equal weights. As many diagnostic characters as possible are used for numerical analysis, and these are formulated as yes (+) or no (-) alternatives. In conventional identification, a particular yeast is identified by matching the observed cultural, morphological and physiological characteristics with those recorded in standard monographs such as those of van der Walt and Johannes (1984) for yeasts previously described. A set of 58 or so characters are examined together in order to narrow down the possible classification of an unknown yeast strain.

The idea of phenetic classification is based on characters of equal weight and it originates from the eighteenth century French botanist, Michel Adamson, and now we can apply the term 'adamsonian' to phenetic classification. During the past twenty-five years a large number of publications have appeared concerned with what has become known as numerical taxonomy.

Normally the term Numerical Taxonomy is applied to systems of classification which are basically 'adamsonian' but in which the degree of similarity of organisms is assessed in quantitative rather than merely qualitative terms, Campbell (1974).

There are many advantages in having some numerical estimate of the degree of phenetic similarity or difference between a pair of yeasts of which the most obvious is that it can provide a rational basis for deciding the levels of taxonomic rank.

In statistics, the search for relatively homogeneous groups is called cluster analysis (numerical taxonomy).

In yeast taxonomy, cluster analysis is used to classify yeasts. The aim of cluster analysis is to identify homogeneous yeast groups or clusters.

The concept of distance and similarity are basic to many statistical techniques. Distance is a measure of how far apart two yeasts are, and similarity measures closeness. Distance measures are small and similarity measures are large for yeasts that are similar. In cluster analysis, these concepts are important since cases are grouped on the basis of their 'nearness', Everitt (1980).

It was shown by Sneath (1957 b) and others that there was at least as much difference between pairs of species of certain established genera as there was between pairs of genera.

There are several distinct approaches to cluster analysis (numerical taxonomy), Hughes (1978) but all start by:

- a) Collecting the organism, or groups of organisms, to be compared, which are now known as Operational Taxonomic Units (OTUs).
- b) Observing these OTUs for the presence or absence (1,0) of quantity of a large set of characters, say 78 characters;
- c) Calculating distances or similarity coefficients;
- d) Forming clusters - criteria for combining clusters;
- e) Performing a cluster analysis.

A character is usually defined as an attribute about which a single statement can be made e.g. 'present (+ or 1) or absent (-or 0) or some quantitative measurement.

Some characters may not be permissible because they are redundant i.e. are expressions of an already listed character. For example if an OTU ferments both glucose and sucrose with the formation of acid and gas this may appear to generate three distinct characters, namely, acid from glucose, gas from glucose, sucrose fermented. It is, however, invalid to score "gas from sucrose" as a separate character since we know that the fermentation of sucrose involves an initial hydrolysis to glucose, which is subsequently fermented to acid and gas Hughes (ibid). It is essential to the principle of Numerical Taxonomy that each of the OTUs should be examined across the complete set of characters, so that true comparisons may be made.

Care must be taken not to make comparisons that are illogical. Suppose that one OTU ferments glucose to acid and gas, whereas a second OTU does not ferment glucose at all.

In the case of the first OTU we may score a positive character for each of the attributes. However, with regard to the second OTU we may score a negative character for lack of production of acid from glucose, but it is now illegal to score a result for "gas production" since this depends on the prior formation of acid which we have already noted as absent. We therefore score "NO COMPARISON" (NC) rather than (-) or (0) for gas production by OTU number 2, which means that this character cannot be used when comparing the similarity of OTU number 2 with any other OTU.

Sneath and Johnson (1972) investigated the effect of experimental errors of microbiological tests upon similarity values used in cluster analysis: Their findings prompted questions of practical considerations, Hughes (ibid), such as:

1. Since observations of characters is necessarily carried out under the artificial conditions of the laboratory, can we make a true comparison of micro-organisms which might behave differently in their "natural" environment?
2. If we have among the OTUs some organisms that can carry out certain reactions at one temperature of incubation but not at a higher in comparison with organisms that can carry out the same reaction only at the higher temperature, should we then use different temperatures of incubation in order to correctly characterise the different OTUs?

A method has been developed by Sneath (1968) for calculating taxonomic relationships so as to reduce dissimilarity that is due principally to differences between the strains in growth rates or other similar factors.

By this the total difference is partitioned into two components "Vigour Difference" and "Pattern Difference" which are analogous to Size and Shape difference in higher organisms. (e.g Angiosperms and Vertebrates).

Sneath (ibid) states that a problem in the numerical taxonomy of micro-organism which have received little attention is whether differences in growth rates, incubation temperatures, etc... may exaggerate phenetic dissimilarities by introducing difference that can be considered as due to unwanted common factors. The taxonomist wishes to discount, for example, those differences that can be plausibly explained by unequal growth rates of strains. This may be a major factor in studying groups (clusters) in which fast-and slow-growing variants (OTUs) are common, or where different OTUs differ markedly in rates of growth. Wayne (1967) notes that it is difficult to read the tests if both fast- and slow - growing OTUs are studies together. Temperature , pH, composition of the basal medium and the like, may raise the same problem.

It is commonly found that a slowly growing OTU gives weak or negative reaction in a test read at the usual time, while on longer incubation it becomes clearly positive. It is uncommon for position tests to revert to negative on continued incubation at least for moderate incubation times, Sneath (ibid). Some exceptions do occur, especially in carbohydrate fermentation tests, but usually the number of tests giving positive reaction at any given time of reading is a partial measure of growth rate, and more generally of metabolic activity, at least when strains of the same species are under study.

Sneath (ibid) states that there has been little detailed work upon the influence of incubation temperatures and similar factors upon taxonomic character.

At temperatures above the growth optimum the behaviour with taxonomic tests is somewhat erratic (perhaps, because the lethal temperature is only a little higher), but at suboptimal temperatures the general finding is that metabolic activities are usually similar but slower. Tests, therefore, take longer to become positive (and sometimes remain negative even after long periods). A concept is needed which can accommodate different factors like growth rate, incubation temperature, etc.. The term VIGOUR has been suggested for this wider concept of general metabolic activity or achievement. Vigour represents the ability of an OTU to carry out varied metabolic activities as shown by its performance in a given set of tests. The concept vigour depends on the set of tests and the conditions, and that the number of positive features is characteristic of different species of OTU. Another difference, PATTERN, is that which is found between equally vigorous strains as being composed of two components, Vigour Difference and Pattern Difference.

As an example, a mouse and a rat are very similar in shape but the rat is about double the size of a mouse. As measured by a taxonomist the difference (the distance, size) would be large, although the shape difference would be fairly small. So the Pattern Difference is the more important in cluster analysis. There are various ways of dissociating shape from size, such as using proportions or correlation coefficients.

One can separate vigour (the analogue of size) from Pattern (the analogue of shape) as follows: A vigorous OTU will show more positive test results than a less vigorous one. Furthermore, there will be few tests in which the vigorous OTU scores as negative but the less vigorous one scores a positive.

Vigour implies a degree of metabolic achievement rather than a rate of metabolic activity, Sneath et al (ibid). An OTU will usually show higher vigour after long incubation than after short incubation, because more tests will be positive after the longer time. Vigour is the proportion of positive tests.

The degree of relatedness between OTUs is a function of the number of similar characters in proportion to the total number of characters examined. The similarities between pairs of strains is then expressed by a similarity coefficient or S value, which is defined as,

$$S = \frac{a + d}{a + b + c + d} = \frac{a + d}{n},$$

where a and d are the sums of the characters which are common to OTU A and OTU B (a both positive, d both negative); b is the sum of the characters in which A is positive and B is negative, and c is the sum of the characters in which A is negative and B is positive, giving the familiar 2 x 2 table

		OTU B	
OTU A	+	a	b
	-	c	d

Vigour and pattern coefficients have been used to calculate relations based on positive (+) and negative (-) responses so as to reduce dissimilarity that is due principally to differences between yeast strains (OTUs) in growth rates, biomass yield or fermentation rate (CO₂ and ethanol yield).

By this the total difference is partitioned into two components: (Vigour Difference)² or $\left(\frac{c-b}{n} \right)^2$ and

Pattern Difference or $\left(\frac{4bc}{n^2} \right)$.

The concepts have been applied to ethanol tolerance effect of lactic acid on growth and fermentation; effect of glucose concentration on growth and ethanol production.

Yeasts have extensive adaptability determined by their small size. A cell has space for only some 100,000 molecules of protein, Hans (ibid). Enzymes that are not currently being used cannot be held in reserve and therefore certain catabolic enzymes are produced only if the appropriate substrate is present. Such inducible enzymes can constitute up to 10% of cellular protein under some conditions, Hans (ibid).

This means that regulatory mechanisms play a far greater part in yeast cells and are more easily recognizable than in other organisms.

As a solution to the almost flux state of yeasts it is generally considered best to compare yeasts OTUs over a rigidly standardized set of tests. It is appreciated that certain of these conditions will introduce bias when measuring the degree of similarity between pairs of yeasts OTUs.

This course of action is adopted for two chief reasons, Hughes (ibid).

1. Practical (or pragmatic) expediency;
2. If a wide range of characters are observed the bias should be 'diluted' out. In general at least 70 characters (cultural, morphological, and physiological) are used.

Care is taken such that tests are not used to generate characters when it is known that bias is inherent in the test condition. For example, we may adjust the sensitivity of a test for aerobic fermentation so that it is read as positive only with those yeast OTUs that we call aerobic fermenters. To use this test in a phenetic comparison of aerobic fermenting yeasts would obviously introduce bias since we have prejudiced the issue by distinguishing certain yeast OTUs as aerobic-positive before hand. Such a test, however, would be valid if applied to unrelated yeasts, e.g. those yeasts that are NO_3^- positive against those that are NO_3^- negative, or asco-genos against asporogenous yeasts.

It follows, therefore, that comparisons of OTUs based on a large number of characters are likely to be more accurate (free of bias) than comparisons based on only a few characters.

Further, special difficulty may exist when an attempt is made to compare organisms that have very different growth rates under standardized conditions e.g. highly fermentative Saccharomyces cerevisiae and Brettanomyces. Here, there is clearly the possibility of bias due to comparison of characteristics that depend on metabolic rate when for the slower-growing yeast strains.

The solution here would be: a) to prolong incubation period so that the reactions of the slower grower are realized, when fast-growing yeast starts to feel stress under accumulating wastes or, b) we have recourse to special methods of calculations that attempt to separate effects due to Vigour from the due to Pattern, Sneath (1968).

After an OTU character table (matrix) has been compiled and all possible pairs of OTUs are compared and their similarities computed, there are four basic methods by which measures of similarity may be computed. These are:

- 1) Correlation coefficients;
- 2) Measures of taxonomic distances, d ;
- 3) Similarity coefficients

The first two methods have the advantage that the characters which are expressed as quantitative data may be more or less directly incorporated into the calculations of similarity. The correlation coefficients are closely related to the commonly used statistic, r , which expresses the degree of correlation between two sets of bivariate data and can vary from +1 (absolute correlation) to -1 (absolute negative correlation).

Thus, two yeast strains that were absolutely identical in all characters studied would generate a coefficient of +1, two yeasts that were absolutely opposite in every character (if this were possible) would generate a coefficient of -1, whereas a coefficient of 0 would indicate no correlation of the characters of the first yeast with those of the second.

S, Vigour and Pattern coefficients measures the overall similarity between a pair of yeast OTUs by the proportion of features in which they match. The advantage of these coefficients in the present study is that they can apply to the quantal (+, -) or (yes, no) character states.

So, there is no need of coding as required in quantitative observations. These coefficients produce acceptable and self-consistent classifications, Sneath (ibid).

CLUSTER ANALYSIS

After numerical estimates of the degree of similarity between possible pairs of yeast OTUs (i.e. $\frac{n!}{r!(n-r)!}$) have been generated, the next step is to form the groups (or clusters) which are the basis of the final classification, an operation known as CLUSTER ANALYSIS.

After numerical estimate of the degree of similarity between all possible pairs of OTUs have been generated, the next step is to form the groups or clusters which are the basis of the final classification.

The coefficients referred to above may be clustered in two ways, Everitt (1980):

1. Hierarchical cluster analysis,
 - a) Agglomerative hierarchical clustering,
 - b) Divisive hierarchical clustering.
2. Nonhierarchical clustering methods.

There are many criteria for combining clusters. These criteria are used for deciding which cases, or clusters should be combined at each step. All these criteria are based on a matrix of either distance or similarities between pairs of cases.

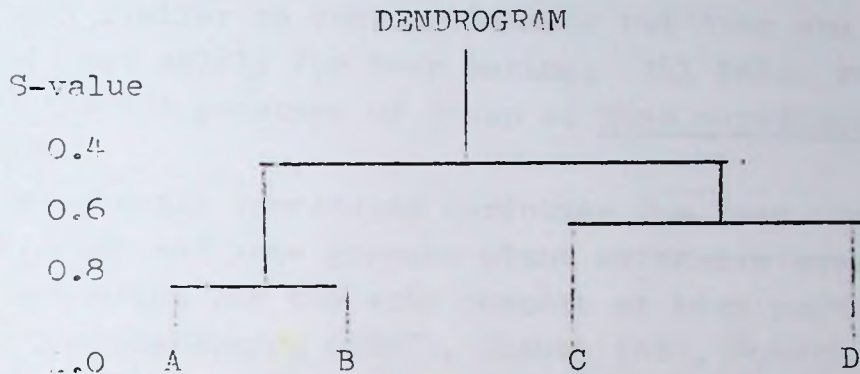
The coefficients may be clustered in three ways: by

1. Single Linkage;
2. Average Linkage;
3. Total Linkage.

The methods lead to computer Programming for the analysis.

When all clusters have been formed at the various levels of similarity, it is usual to present the result in the form of a dendrogram which resembles the diagrams of hierarchical classification or dichotomous

classification but differs in having estimates of similarity rather than qualitative criteria to determine its branching points. Thus



Suppose that at a certain level of similarity (say $S=0.8$), see diagram above, there exist two distinct clusters A and B. When we examine these clusters at a lower level of similarity (say $S=0.6$) we may find a tendency for A and B to fuse into a single cluster. For fusion by

Total Linkage, each OTU in A must have $S \geq 0.6$ with every OTU in B. For fusion by Single Linkage it is sufficient that one OTU in A has $S \geq 0.6$ with a single OTU in B, so making the "single" link between the two clusters, Hughes, (ibid).

Chapter 3

MATERIALS, METHODS AND EQUIPMENT

Part I

Materials (More details are given in the Appendix).

Fruits included

Bananas: ripe beer bananas; varieties of this group of bananas are in many ways similar to cooking bananas but they are almost invariably used solely for beer making. All these varieties probably belong to the AAA genotype of group of Musa paradisica, Purseglove J W (1991).

The use of recently introduced varieties for beer (tonto) making is on the increase and some growers plant extensive areas to pure stands of these varieties for the sole purpose of beer production. The varieties include Bogoya (ABB?), Kisubi (AB), Sukari (AB), Balukule and Kivuvu (ABB), groups of Musa sapientum.

2. Passion fruit, Passiflora edulis. Passion fruits are cultivated for their edible fruits. The pulp and seeds contain approximately: water 72.1%, protein 2.4% fat 2.8% carbohydrate (sugar) 17.3%, mineral salts 1.2% and it is a good source of vitamin C. The juice is used for making soft drinks.

3. Pawpaw, Carica papaya: The edible portion of the flesh contains: water 88%, sugar 10% protein 0.5%, fat 0.1% acids 0.1%, salts 0.6%, and rich in vitamin A and some vitamin C.

4. Mango, Mangifera indica. The edible portion of ripe fruit is 60 - 75% by weight. Water 15%, protein 0.5%, sugar 10%; rich in vitamin A and B. The above figures are sited from Purseglove(ibid).

Grains included:

1. Maize, Zea mays. 100g of whole grain contain 10g protein (poor in tryptophan and over-rich in leucine) and 4 g fat, and provides 360 calories; the germ (embryo) contains 12% by weight of the whole grain and has 22% of the total protein and 84% of the oil. Germinating maize grain has nearly all the starch converted to malt sugar (maltose).

The above data are from Jameson (1970).

2. Sorghum, Sorghum bicolor

3. Finger millet, Eleusine coracana.

Beverages included: ((Detailed account ,see Appendix)

1. Tonto
2. Kwete
3. Amarwa
4. Muramba

Thus, the localities, namely Central, Western, Eastern and Northern Uganda, provided opportunity to the researcher to examine ecological and anthropogenic factors in the understanding of the impact and significance (socio-economic) of brewing processes on the distribution of yeast strains in Uganda.

Origins: Tonto from Central Uganda, Muramba from Kigezi, Ajon/Amarwa from North-East of Uganda.

Part II METHODS AND EQUIPMENT

Collecting of Samples

Yeasts were known, through literature, to occur on surfaces of ripe fruits, grains, including malts used in preparation of Uganda's "beers" and "wines". It was further known that yeasts were involved in the fermentation of the "beers" and "wines".

Therefore, the following procedures were employed.

Fruits:

The samples were collected from the field as follows: 10 random samples of ripe banana fruits were cut off from the cluster with sterilized blade and placed immediately in clean cellophane bags and tied, and were well labelled with date, time, location, variety of bananas. The fingers were picked up by sterilized forceps. Similarly, passion fruit (10 samples), pawpaw (10 samples), and pineapples (10) were collected from plants with sterile gloves. The bags were labelled as follows: Masaka/Rakai fruit samples as an example: $MR_{ij}B_y$. (More details on coding, see Appendix)

- $R_{1j}B_1$ = sample for Kisubi, from Masaka, village j, not named here.
 $R_{2j}B_1$ = sample for Kisubi from Rakai
 $R_{1j}B_2$ = sample for Bogoya from Masaka.
 $R_{2j}P$ = sample for pineapple from Rakai, etc....

Grains

The hundred grams of each of sorghum, millet and maize from the same localities were collected in sterilized jars and appropriately labelled, e.g. $MR_{1j}SO$, a sample of sorghum collected from Masaka.

Beverages

Ten 75 ml sterilized bottles with screw tops were used to collect ten samples each of tonto, kwete, amarwa (ajon) and muramba. These fermenting beverages (24 hours of fermentation) were always available in the villages visited in Masaka, Rakai, Mbarara, Ibanda, Rukungiri, Jinja, Tororo, and Mbale-Soroti, on special order.

Time was spent interviewing people and observing how beverages were prepared.

The materials (samples) from the field were brought to the laboratory. Bags, jars and bottles with their contents were stored in the laboratory in the Department of Botany, Makerere University at 0-4C, in the refrigerlator.

Methods and Equipment

Isolation and Identification, and maintenance of yeast stocks.

a. Isolation of yeasts:

Media: Four different media were used for the isolation and maintenance of yeast cells. These media were: (more details are given in the Appendix).

1. Banana-juice yeast extract agar (BYA).
2. Yeast extract - peptone - glucose agar (YPAG).
3. Nutrient agar-glucose (NAG).
4. Yeast extract malt extract agar (YMA).

The following technique was used in making serial dilutions. Ten ml extract from 100g grain surface, using sterile water; 10 fruits or 10ml of fermenting tonto, kwete, muramba and amarwa (ajon) were plated as follows. The extract - dilution method consisted of taking 1 ml of the extract and then obtaining a progressive series of dilutions with a dilution factor of 0.1 or 1/10. From one or more of the dilution, 1ml samples are placed in sterile petri-dishes and dispersed with melted but cooled agar (at about 37 C) by shaking and rotating the plate before the agar solidified.

By trial and error various dilutions were used, from grain and fruit surfaces and from beverages. The dilutions used, usually between 1/1000 and 1/100,000 were chosen so that the degree of interference between developing colonies on the plates through competition and antagonism could be minimized, Warcup (1955 b, 1957). Waksman (1927) recommended 35-45 colonies per plate. Since yeasts are unicellular organisms it means each colony may be considered to have originated from an original individual yeast cell active on the surface of the grain or fruit or in the beverages.

rains or fruits, of known weight or surface area, respectively, were placed in sterile tap water in a sterile jar. The jar was shaken by hand for 15 minutes. Serial dilutions (1/1000) were then plated.

isolation: For each medium, 25 ml each was dispensed onto sterile petri-dishes for cultivating and isolation of yeasts, and 10 ml each was dispensed into McCartney tubes for agar slants for the maintenance of pure cultures. Cultures diagnosed as pure by cultural and morphological diagnosis, were initially streaked onto BY4 and PGA and incubated at room temperature for 18 - 24 hours or 48 hours, sometimes up to 7 days or 1 month, for slow-growing yeasts. Yeast colonies which showed different features were transferred onto medium YMA for further purification. Finally the pure cultures were maintained on slants made from NAG medium.

Cultures on slants were tested for cell viability using 1% methylene blue solution and a haemocytometer or replating and counting colony forming units (c.f.u).

Yeast colony diagnosis involved studying colour, surface texture (matted, rough, smooth), degree of glossiness, colonial cross-section (flat, raised); margin whether smooth or dented; consistency (mucoid, friable, coherent), and cell morphology: mycelium (pseudomycelium; true mycelium); manner of cell division (binary fission; budding: bipolar or multipolar); cell shape (globose; oval; kidney-shaped; ellipsoidal; cylindrical; filiform; fusoid). The pure isolate were marked $MR_{ij}BY_z$, etc....., where Y_z stood for yeast strains isolated from Masaka on surface of bananas. Tentative isolation of colonies that were intermediate between yeast, bacteria and moulds was made and confirmed by microscopic examination (X40 mag). A loopful of culture picked up as representative colony was streaked to a plate of YMA or NAG for purification. Three successive isolations were made from the representative colony. It was observed that not all yeasts with the same cultural characteristics belonged to the same strain. Two colonies, cream in colour, glossy with dented margin, one may form a pseudomycelium and the other without pseudomycelium. In fact, their physiological characteristics may differ quite widely. The converse was true.

However, the number of colonies was so large, at times, that only a representative of each colony could be isolated and purified for characterisation.

It was important to examine microscopically the primary isolation plates because some characteristics (cultural and morphological) changed on subculturing, especially on different media.

All 218 isolates were randomly made. These were reduced to 38 after diagnosis and primary characterisation. These were labelled to Y₂₁₈.

b) Identification

Two methods of analysis were used.

The conventional method for yeast identification described by van der Walt (1984), Barnett et al (1983), Krag-van Rij (1984) and Jeff and Starmer (1987), Lodder (1970).

Numerical Taxonomy of genera of yeasts:-

The aim was to examine the correlation (clustering) between taxonomic groups and potentially useful properties of yeasts for identification. Cluster analysis (procedure CLUSTER) or Numerical Taxonomy should reveal strains of yeasts with similar morphological and physiological characteristics so that one could be able to target future marketing or biotechnological strategies more efficiently. Each strain is characterised in terms of morphological and physiological tests.

CONVENTIONAL METHODS OF IDENTIFICATION

Cultural and morphological characters

TABLE 1 Scheme of rapid identification (Codes for sources are given at the end of the table)

Sample code		
MR-11-Z-Y1	B,P,M,PA,PW,TO,KW,AM	glistening; pellicle; budding bipolar
MR-11-Z-Y2	- do -	cream, pellicle thin; cells ovoid
MR-13-Z-Y4	B,P,TO	cream, smooth, cells elongate
MR-22-Z-Y5	B,PA,TO	cream, ovoid, with a ring
MR-12-Z-Y7	B,P,PA,PW,TO	white, budding
MRU-13-Z-Y8	TO	varied from thin pellicle to folds
MRU-11-Z-Y9	B,P,PW,TO	smooth, cell ovoid
MR-12-Z-Y10	TO,KW	folded pellicle, pseudomycelium
MR-22-Z-Y14	B,P,M,PA,PW,TO,KW	with a ring, bipolar budding
MR-14-Z-Y22	- do -	cells elongated, bipolar
MRU-11-Z-Y23	- do -	budding, pseudomycelium absent

12.	MR-21-Z-Y28	-do-	mat, entire, pellicle
13.	MRU-11-Z-Y32	-do-	tree-like structures, spheroidal
14.	MR-24-Z-Y38	-do-	mycelium, spheroidal
15.	MR-11-Z-CaY33	-do-	pseudomycelium
16.	MR-14-Z-CaY6	-do-	" with blastospores
17.	MR-22-Z-CaY60	-do-	pellicle wrinkled
18.	MR-22-Z-CaY50	-do-	cells ovoid
19.	MR-21-Z-CrY11	-do-	produced amylose
20.	MR-22-Z-CrY37	-do-	produced amylose cells round; ovoid
21.	MR-11-Z-DeY13	-do-	dull pellicle, round, short 2x6
22.	MR-11-Z-DeY46	-do-	cells 4 x 6
23.	MR-12-Z-HaY14	-do-	dry pellicle, wrinkled
24.	MR-13-Z-DeY48	-do-	cells round,
25.	MR-12-Z-HaY17	B, P, PA, PW TO	pellicle, bipolar budding
26.	MR-12-Z-HaY49	B, P, M, PA, PW, TO, KW MA	round,
27.	MR-13-Z-HaY62	"	budding, cream, smooth
28.	MR-13-Z-HaY67	"	glistening
29.	MR-11-Z-HaY20	"	entire, white
30.	MR-22-Z-HaY68	"	cream
31.	MR-11-Z-SaY21	"	cream
32.	MR-11-Z-SaY25	"	greyish white; round; ovate
33.	MR-21-Z-SaY12	"	ellipsoid, multilateral budding
34.	MR-12-Z-ScY26	"	white colonies, cylindrical cells
35.	JT-11-Ca2	AI, AM, SO, MI, MAI	
36.	JT-12-Z-Cal1	"	
37.	JT-11-Z-Han62	B, AI, AM, SO, MI, MAI	
38.	JT-22-Z-Han32	AI, AM, SO, MI, MAI	budding, many clusters, oval
39.	JT-22-Z-Sa30M	B, P, AM, SO, MI, MAI	
40.	JT-13-Z-Sa30N	B, AM, SO, MI, MAI	round
41.	JT-22-Z-Sa30P	"	
42.	JT-11-Z-Sc44N		
43.	JT-13-Z-Sc44P	AM, MAI	
44.	JT-Z-Sc44T	AM, MAI	
45.	JT-Z-Cr16	AI, AM, SO, MI, MAI	
46.	JT-12-Z-De41	B, P, PA, AI, AM, MI, MAI	
47.	MR-22-Z-RhY24	B, P, M, PA, PW, KW	Yellow, to red
48.	MRU-12-Z-CrY57	B, P, M, PA, PW, TO	
49.	MRU-11-Z-CaY42		
50.	MRU-11-Z-HaY49		
51.	MRU-22-HaY62		
52.	MRU-13-Z-HaY67		mostly budding

- | | |
|-------------------|-----------------------|
| 3. MRU-11-Z-SaY30 | oval, few in clusters |
| 4. MRU-11-Z-SaY68 | budding, large 5 x 7 |
| 5. MRU-22-Z-ScY56 | cylindrical |
| 6. MRU-13-Z-CrY57 | clusters of 2 - 4 |

Codes

MR ZY : MR. stands for locality in Masaka/Rakai
ij For i = 1, then MR, stands for a locality in Masaka;
i = 2, a locality in Rakai
j = stands for a specific village where the sample was taken.
Z = stands for the source of yeast e.g.
Z = can be B,P, TO etc..
If Z is a fruit, we have
B = banana, P = Pineapple,
M = mango, PW = Pawpaw,
If Z is a beverage; we have TO = tonto, KW=Kwete,
AM = Amarwa or Ajon;
If Z is grain, then
SO = Sorghum
MAI = Maize
MI = Millet
AI = Ainyeles,
Y = yeast strain
Ca = Candida, Cr = Cryptococcuss
D = Debaryomyces
Ha = Hansenula
Han = Hanseniaspora
Sa = Saccharomyces
Sc = Schizosaccharomyces
Rh = Rhodotorula

CONVENTIONAL TAXONOMY: Detailed analysis

Schizosaccharomyces pombe

A) Cultural characteristics

i) Growth in liquid media

Observations were made on malt extract broth. The species, after 72 hours at 23 C formed a sediment.

ii) Growth on solid media

Observation were made on malt agar; yeast extract-malt extract agar; potato agar. The streak on the surface of the medium was narrow, creamy or white going brown with time (4 weeks). Growth was generally slow. The culture was raised (central part) and irregular. It had a brittle consistency.

b) Morphological characteristics

After 72 hours at 23 C cells were observed to be ellipsoidal, septate. Some parts were seen broken up into arthrospores. The cylindrical mycelial cells were about 20-40 m in length and 3-5 μ in diameter. see Figure 4

c) Physiological characteristics

Fermentation of glucose, D-galactose, maltose, sucrose, raffinose and melibiose was observed during the first two days (48 hours) of growth. Growth in No_3 , vitamin-free media was negative. Maximum growth temperature on malt was 43C. Starch test was positive.

Discussion

Origin: the species was isolated from all sources examined.

This culture after maintenance on malt agar, dilution plates on the same medium gave rise to whitish powdery colonies which grew rapidly across the surface. The pseudomycelium was more fragmented and cells more or less cuboidal rather elongated cylinders.

Saccharomyces cerevisiae

a) Cultural characteristics

i) Growth in liquid media: Observations were made on malt extract and 2% glucose-yeast extract broth; after 3 weeks at 23C a sediment and a pellicle were present with a ring.

ii) Growth on solid media: Observations were made on malt agar; yeast extract-malt extract agar; potato agar. The streak culture was butyrous, friable, cream coloured, raised. After one month at 23 C the culture became brownish-cream.

a) Morphological characteristics

This species showed very large variation in morphological characteristics. Cells were round, ovate, elongated, often formed a pseudomycelium with multipolar budding.

In young cultures 24 - 72 hours at 23°C the dimensions were 4-14 x 3 - 7 μ m. Figure 17.

b) Physiological characteristics

The species was noted for its efficiency in fermenting sugars yielding ethanol and carbon dioxide. It fermented glucose, galactose and maltose very well. Raffinose was weakly fermented. Growth in KNO_3 was negative; the species grew weakly in vitamin-free broth. The growth on 50% (w/w) glucose - yeast extract was reasonable; there was little growth on 60% (w/w) glucose yeast extract.

Discussion

S. cerevisiae was the yeast frequently isolated from tonto and ripe banana. The yeast showed great variation in morphology. Therefore, cell length proved to be of little diagnostic value. However, cell width was fairly characteristic of the species and could be considered dependable.

Saccharomyces chevalieri

a) Cultural characteristics

i) Growth in liquid media: Observations were made on malt extract and 2% glucose-yeast extract broth. After 2 weeks a sediment was formed.

ii) Growth on solid media: After 72 hours at 23°C observations were made on malt agar; yeast extract - malt agar. The streak culture was butyrous, cream to grey; flat.

b) Morphological characteristics

After 72 hours at 23°C the cells were spheroidal, ovoid or cylindrical, (2.5 - 7 x 4 - 10) μ m. The cells occurred singly or in pairs. A few times the yeast was observed to form chains, Figure 6.

c) Physiological characteristics

Glucose and sucrose were fermented, but galactose, maltose and lactose were not fermented. There was no growth in KNO_3 .

Saccharomyces pastorianus

The species was isolated from tonto.

) Cultural characteristics

) Growth in liquid media: Observations were made on malt extract and 2% glucose-yeast extract broth. After 4 weeks at 23°C a sediment was formed, with a ring.

i) Growth on solid media: After 72 hours the streak culture was butyrous, cream coloured, shiny; margin undulating.

) Morphological characteristics

After 72 hours at 23°C the cells were observed to be spheroidal, ovoid or elongate, 3 - 7 X 4 - 17 μ m. They occurred singly, in pairs short chains or small clusters, Figure 1

) Physiological characteristics

At first the species failed to use galactose, after one week; later the species grew well in galactose. The species was isolated from Tonto.

Saccharomyces ellipsoides

This species was not very different from Saccharomyces cerevisiae. The species was often isolated from Tonto; banana, pineapple and mango surfaces. It had a higher tolerance of ethanol. Cells were ellipsoidal to ovoid, 6-8 X 5-6 μ m

Saccharomyces capensis

) Cultural characteristics

) Growth in liquid media: Observations were made on malt extract and 2% glucose-yeast extract broth. After 4 weeks at 23°C a sediment was present and a ring.

i) Growth on solid media: After 4 weeks observations were made on malt agar; yeast extract-malt agar. The culture was cream, smooth, shiny. The margin was entire. The streak was butyrous.

) Morphological characteristics

After 72 hours at 23°C on morphology agar and broth cells were observed to be spheroidal, ovoid, at times cylindrical, 3-7.5 X 3-9 μ m, formed rudimentary pseudomycelium. Figure 1

) Physiological characteristics: After 72 hours, there was no growth in maltose. Later (after 3 weeks) the species grew well. So there was delayed utilization of maltose; showed vigorous fermentation of glucose, sucrose and weakly fermented raffinose.

ekkera bruxellensis

) Cultural characteristics

- i) Growth in liquid media: A pellicle and a sediment was formed; developed an aromatic odour.
- ii) Growth on solid media: After a week at 23°C the streak was shiny, smooth, cream coloured.

) Morphological characteristics

Pseudomycelium was formed; vegetative cells varied in shape; ovoid; ellipsoid or elongate. After 72 hours at 23°C the cells measured 4 - 5 X 6 - 10 μ m.

) Physiological characteristics

Figure 5

KNO₃ was positive.

Lodderomyces elongisporus

) Cultural characteristics

-) Growth in liquid media: A sediment was formed.
- i) Growth on solid media: The streak culture was butyrous, friable, cream coloured, flat, spread, with entire margin. Produced esterical aroma.

) Physiological characteristics

Figure 3

The species was strongly fermentative; KNO₃ was negative. The species was frequently isolated from tonto.

Other strains were coded for cluster analysis because the available monographs could permit identification only to genus.

The strains Candida Y42, Candida Y50, Debaryomyces Y46, Debaryomyces Y42, Candida Y33, Candida 2, Candida 11, Hansenula Y49, Hansenula 32, Hansenula Y67, Hansenula Y62, Hanseniaspora Y68, Hanseniaspora Y20, Saccharomyces Y30, Saccharomyces Y68, Saccharomyces Y30, Saccharomyces Y68, Saccharomyces Y21, Saccharomyces Y25, Saccharomyces Y12, Saccharomyces 30M, Saccharomyces 30N, Saccharomyces 30P, Schizosaccharomyces Y56, Schizosaccharomyces Y26, Schizosaccharomyces Y26, Schizosaccharomyces 44M, Schizosaccharomyces 44N, Schizosaccharomyces 44T, Cryptococcus Y57.

Other strains could not even go to genera level of identification. These were coded as: Y1, Y2, Y4, Y5, Y7, Y8, Y9, Y10, 14, Y22, Y28, Y32, Y38. In all cases, the strains were fully characterised and tabulated in Tables II -XII.

-67-

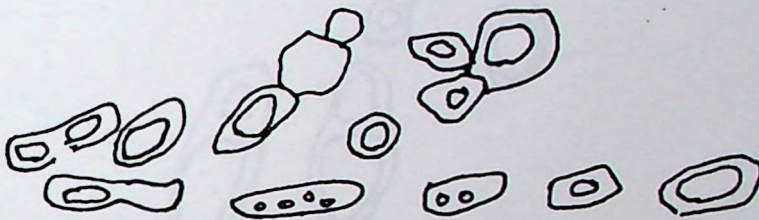


Fig 1 Dekkera bruxellensis after 96
hours on YMA

-68-

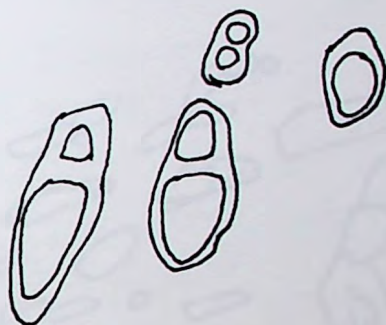


Fig 2 Saccharomyces cerevisiae after 72 hours
on YMA

-69-

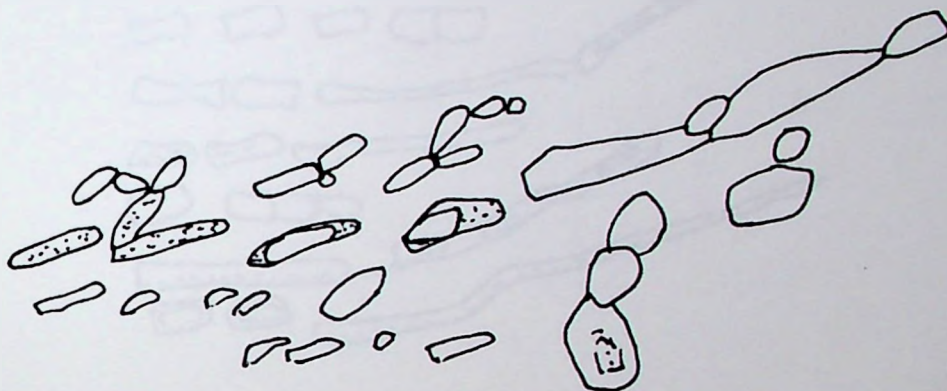


Fig 3 Lodderomyces elongisporus after 72 hours
on YMA

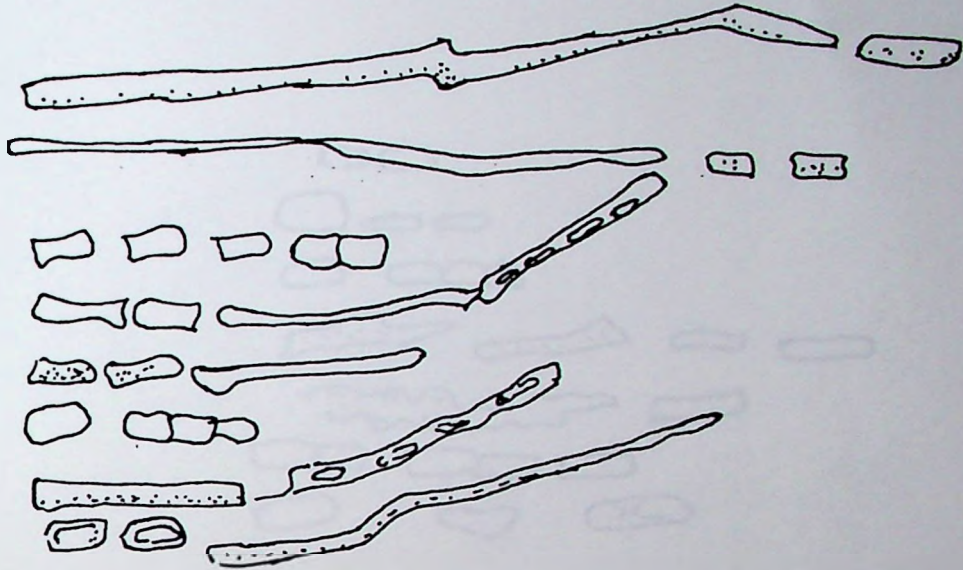


Fig 4 Schizosaccharomyces pombe after 96 hours
on YMA



Fig.5 Debaryomyces hansenii after
72 hours on YMA

-72-



Fig 6 Saccharomyces chevalieri after 72 hours
on YMA



Fig 7 Saccharomyces capensis after 48 hours
on YMA

Chapter 4 : Results

TABLE II

YEASTS ASSOCIATED WITH FRUITS, BEVERAGE AND GRAINS IN MR_{ij}

	B	P	M	PA	PW	TO	KW
<u>Fermentative Yeasts</u>							
(Totals in %)							
Candida Y50	74.9%	3.6	11.2	2.7	2.2	4	1.3
Candida Y33	63.6	2.2	12	4	8.7	6.4	2.7
Candida Y6	48	5.2	24	6	5.3	7.5	3.8
Candida Y60	70.7	1.8	14	3.7	3.7	4.9	1.2
Hansenura Y4	69.8	7.4	8.6	2.5	4.3	5.6	1.8
Hansenura Y17	72.8	5.2	12	3.5	3.5	2.9	0
Hanseniaspora Y68	59	2.6	21	58.8	4.6	3.9	2.6
Hanseniaspora Y20	68	2.8	19.9	2.2	3.6	3.3	3.9
Saccharomyces Y21	62.5	5.7	15.3	7.3	1.1	4.5	3.4
Saccharomyces Y25	67	12	9	6	1.5	3	1.5
Saccharomyces Y12	70.5	1	19.5	2	4	1.5	1.5
Schizosaccharomyces Y26	71.7	3	13.6	5.5	3.5	1	1.5
<u>Non-fermentative Yeasts</u>							
Cryptococcus Y11	36.9	12.8	19.8	8.16	12.82	4.66	5.80
Cryptococcus Y37	23.4	11.2	12.1	11.2	15.1	13.2	14
Debaryomyces Y13	48.8	7	16.8	4.6	8.4	8.4	6
Rhodotorula Y15	34	14.9	35.5	9.2	3.5	2.1	1
Rhodotorula Y24	48	15.4	10.3	14	11.3	0	1
Torulopsis Y29	63.3	16.3	10.2	5.4	1.8	1.8	1.2

Key: B = Banana
P = Pineapple
M = Mango
PA = Passion Fruit
PW = Pawpaw
TO = Tonto
KW = Kwete

TABLE III

YEASTS ASSOCIATED WITH FRUITS, BEVERAGES & GRAINS IN MRUI

	B	P	M	PA	PW	TO	KW
Fermentative Yeasts (totals in %)							
Candida Y42	60.2	7.3	22.2	2.1	2.1	2.1	3.8
Candida Y50	50.4	3.1	19.4	13.2	6.25	4	2.3
Hansenura Y49	64.6	9/5	12.2	2.1	2.1	1.6	7.9
Hansenura Y62	34.6	6.1	49.2	3.4	5	1.1	1
Hanseniospora Y67	56	5.8	18.7	13.8	4.9	3.1	2.2
Saccharomyces Y30	64	8.2	11.4	4.4	2.5	716	2
Saccharomyces Y68	59.2	4.9	19.7	7/7	3/5	3.5	1.4
Schizosacc- haromyces Y56	57.5	11.9	22.8	2.7	1	2.7	1.4
Non-fermentative yeasts							
Cryptococcus Y57	33.5	7	45.5	2.00	2.00	8	2
Debaryomyces Y46	29.8	22.4	29.1	4.2	6.9	7.67	0
Debaryomyces Y48	58.6	2.7	20.7	1	4.5	9.9	2.7

TABLE IV

YEASTS ASSOCIATED WITH FRUITS, BEVERAGES & GRAINS

Fermentative yeasts
(totals in %)

Candida 2	0	0	0	0	0	5	70	3	10	12
Candida 11	0	0	0	0	0	4	81	2	8	5
Hansenura 62	2	0	0	0	1	2	80	7	6	4
Hansenura 32	1	0	0	0	0	4	64	5	12	15
Saccharomyces DOM	21	3	0	0	0	0	32	14	10	20
Saccharomyces 30N	24	0	0	0	0	0	30	12	15	19
Saccharomyces 30P	18	0	0	0	0	0	29	13	17	23
Schizosaccharo myces 44M	0	0	0	0	0	6	73	6	7	6
Schizosaccharo myces 44N	0	0	0	0	0	0	79	2	1	9
Schizosaccharo myces 44P	0	0	0	0	0	0	88	2	1	9
Schizosaccharo myces 44T	0	0	0	0	1	5	76	4	6	8

Non-fermentative
yeasts

Cryptococcus 16	24	24	31	8	0	2	1	4	1	5
Debaryomyces 41	37	6	18	4	1	5	15	0	5	9

KEY:

B = Banana (Musa spp)	AI = Ainyeles
P = Pineapple (Ananas sp)	AM = Amarwa/Ajon
M = Mango (Mangifera sp)	SO = Sorghum
PA= Passion Fruit (Passiflora sp)	MI = Millet
AK= Akiria nuajon	MAI = Maize

JT_{ij} : J = Jinj locality; T=Tororo locality; ij: code for villages

TABLE V											
CHARACTERISTICS DISTINGUISHING BIOVARS OF YEASTS ISOLATED FROM FRUITS											
Raj											
	CaY50	CrY37	DY13	HaY4	HanY20	KY10	PY21	RY15	SaY21	ScY26	TY29
Ascospores	-	-	+	-	-	-	-	-	-	-	-
Ferments											
D-glucose	+	-	w	+	+	+	-	-	+	+	+
Cellobiose	-	-	-	-	-	-	-	-	-	-	-
D-galactose	O	-	-	-	+	-	-	-	+	+	O
Maltose	-	-	-	-	-	-	-	-	+	-	-
Raffinose	+	-	+	-	-	-	-	-	-	-	+
Sucrose	+	-	-	-	+	-	-	-	+	-	-
Assimilates											
Glucose	+	+	+	+	+	+	+	+	+	+	+
L-arabinitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Galactose	-	-	+	-	-	-	-	-	-	-	-
D-arabinose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Trehalose	-	-	+	+	-	-	-	-	-	-	-
Cellobiose	+	-	+	+	+	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	O
Erythritol	-	-	+	-	-	-	O	-	-	O	+
Ethanol	-	-	-	-	O	O	-	-	-	-	-
Galactitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-galactose	-	+	-	-	-	-	-	-	-	-	-
D-glucitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Gluconate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-glucose-amine	+	+	+	DH	+	-	+	+	O	+	+
Glucuronate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glycerol	-	-	-	O	+	+	O	-	-	-	-
Myo-inositol	-	-	-	O	O	O	-	D	-	-	+
Inulin	-	-	O	-	-	-	O	O	-	O	-
L-lactate	-	O	O	-	-	+	-	-	+	O	-
Nitrate	-	-	+	-	-	-	-	-	-	-	-
Lactose	-	-	O	-	-	O	-	-	-	O	O
Maltose	-	-	-	-	-	-	+	+	-	-	-
Mannitol	-	O	-	-	O	-	+	+	-	-	-

TABLE V

Melezitose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Me-glucoside	D	D	-	-	-	-	-	+	+	+	O
Raffinose	-	-	-	-	-	+	+	-	+	+	+
Melibiose	O.WD	-	+	-	-	+	+	+	-	-	-
Ribitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-ribose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salicin	-	-	+	-	+	-	+	-	-	+	+
Soluble starch	-	-	w	-	-	+	+	+	-	w	D/W
L-sorbose	-	+	+	-	-	O	+	O/W	O	+	+
Succinate	D	O	-	-	-	-	-	+	-	-	-
Sucrose	-	-	-	-	+	-	+	-	-	-	+
A-a-rehalose	-	-	-	O	O	-	+	+	-	-	+
Xylitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Grows											+
W/O Vitamins	-	O	-	-	-	-	O	O	-	-	O
W/O biotin	O	+	-	O	-	O	-	-	-	O	-
At 30 C	-	-	O	-	-	-	+	+	-	+	-
At 37 C	-	-	+	-	-	+	O	O	+	+	+
At 42 C	O	-	-	-	-	-	O	O	-	+	O
With 50% Glucose	O/D	-	+/D	-	-	O	O	O	O	-	O
With 0.01% cycloheximide	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
With 0.1% cycloheximide	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Produces amylose	-	+	O	O	O	O	O	-	O	O	O

a = MRij. Where MRij = Masaka area: j - specific villages where samples were taken: MR2j = Rakai area.

b = Biovars are referred to by the following abbreviations

Ca= Candida, Cr = Cryptococcus; D= Debaryomyces*

Ha= Hansenula; Han = Hanseniaspore; K = Kloeckera; P= Pichia:

R = Rhodotorula; Sa = Saccharomyces; Sch = Schizosaccharomyces;

T = Torulopsis.

c = MRI jY₂, = unvarying specific characters are given in cases of failure to confirm with published descriptions (monographs)

d" -" = not applicable (for example, not variable in this species;
+ = positive response;

O = negative response; w = weak response;

D = delayed response: ND = test not performed; the test is not necessary for species of level testing.

/ = response varied in repeated tests.

[illegible]

TABLE VI

Lezitose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Le-gluco side	+	O	O	+	+	O	O	+	O	O	O	O	O	+
Finose	w	w	+	w	+	w	+	w	w	w	w	+	+	+
Biose	+	w	+	W/D	w	w	w	+	w	+	+	w	w	O
itol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ribose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
licin	w	O	+	O	O	O	O	O	O	w	O	O	O	+
uble arch														
sorbose	D	D	-	O	+	+	w	O	+	+	D	w	w	+
ccinate	w	w	+	O	O	-	O	w	w	w	+	w	w	O
crose	W/D	W/D	+	w	w	D	w	w	w	+	+	+	+	+
a-tre- lose	O	O	+	+	O	+	O	O	O	O	O	O	O	O
itol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
xylose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ows:														
O tamins	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O biotin	O	O	O	O	O	O	O	O	O	O	O	O	O	O
30 C	+	+	+	+	+	+	+	+	+	+	+	+	+	-
37 C	+	O	O	+	+	O	O	O	O	+	O	O	O	O
42 C	+	O	O	O	+	+	O	O	O	O	+	O	O	+
th 50% ucose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
th 0.01% clohexi de.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
roduces ylose	+	O	O	O	O	O	O	O	O	O	O	O	+	+

-- MRij, where MRij = Masaka area; j = specific villages where samples were taken; MR2j = Rakai Area.

= Bivars are referred to by the following abbreviations: Ha = Hanseniaspora; Ca=Candida, Cr= Cryptococcus; D = Debaryomyces; Ha= Hansenula; Han = Hanseniaspora; K=Kloeckera; P = Pichia; R = Rhodotorula; Saccharomyces; Schizosaccharomyces; T = Torulopsis.

= MRijY_z = unvarying specific characters are given in cases of failure to confirm with published descriptions (monographs)

TABLE VII:

CHARACTERISTICS DISTINGUISHING BIOVARS OF YEASTS ISOLATED FROM BEVERAGES

MRij	CaY33	CaY6	CrY50	DY3	DY60	HaY17	HaY4	HanY68	ScY26	Sac12	SacY21	SacY25
spores	-	-	-	-	-	-	-	-	-	-	-	-
ments												
ucose	+	+	-	w	w	+	+	+	+	+	+(w)	+
lactose	O	O	-	-	-	-	-	+	-	-	-	-
bose	-	-	-	+D	+D	-	-	-	+	+	-	-
ebiose	-	-	-	-	-	+	-	+	-	+	+	+
ose	+	+	-	-	-	-	-	-	-	-	-	-
ose	+	+	-	-	-	-	-	-	+	+	+w	+
inose	+	O	-	+	+	+	+ $\frac{1}{3}$	-	+	+	+	+ $\frac{2}{3}$
biose	-	-	-	+	+w	O	O	-	+	+	+	+
milates												
ucose	w	w	w	+	+	+	+	+	+	+	+	+
lactose	-	-	+	-	-	-	-	-	+	+	+	+
arbose	+D	-	+	+	+	-	-	-	-	-	-	-
ethyl-D- ose	-	-	-	-	-	-	+	+	-	-	-	-
icin	-	-	-	+	+	-	-	+	-	-	-	-
atin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
rose	-	-	-	-	-	O	+	-	+	+	+	+
tose	-	-	-	-	-	O		+	+	+	+	+
biose	-	-	-	+	+	-	-	-	-	+	+	+
obiose	-	-	-	+	+	-	-	+	-	-	-	-
malose	-	-	-	-	-	+	O	-	-	+	+	+
cose	-	-	+	-	O	-	-	-	-	-	-	-
finose	-	-	-	-	w	-	-	-	+	+	+	+w
ezitose	-	-	-	-	-	-	-	-	-	+	+	+
lin	-	-	-	-	-	-	-	-	-	-	+s	-
rch	-	-	-	w	w	-	-	-	-	-	-	-

TABLE VII

Ethanol	-	-	-	-	-	-	-	-	-	+	+	+
Glycerol	+	+	+	-	-	-	-	-	-	+	+	+
Erythritol	-	-	-	+	+	-	-	-	-	-	-	-
Inositol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lactitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mannitol	+	+	0	-	-	-	-	-	-	+	-	-
Acetate	-	-	+	+	+	-	-	-	-	+	+	+w
Succinate	-	-	+	-	-	-	-	-	-	-	-	-
Citrate	-	-	0	-	-	-	+	-	-	-	-	-
Malate	+	0	0	-	-	+	+	-	-	-	-	-
Nitrite	-	-	-	-	-	-	-	-	-	-	-	-
Aspartate	-	-	-	+	+	+	+	-	-	-	-	-
Asparagine	+	+	-	+	+	+	+	-	-	-	-	-
Glutamate	-	-	-	+	+	+	+	-	-	-	-	-
Glycine	-	-	-	+	+	+	+	-	-	-	-	-
Proline	-	-	-	+	+	+	+	-	-	-	-	-
Nicotin	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
Glucosemine	-	-	D	D	D	-	-	-	-	-	-	-
Me-glucose oxide	-	-	-	-	-	-	-	-	+(w)	-	+w	-
Glucose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Flows												
30C	-	-	-	-	-	-	-	-	+			
37C	-	-	0	0	+	-	-	-	+	+	-	+
42C	-	-	-	-	-	-	-	-	+	-	-	-
With 50% glucose	0	0	+/D	+/D	+/D	0	-	-	0	-	-	-
Induces glycolysis	-	-	-	-	-	-	-	-	+	-	-	-

CHARACTERISTICS DISTINGUISHING BIOVARS OF YEASTS ISOLATED FROM FRUITS

<u>Strain</u> <u>MRUij</u> ^d	CaY42	CrY57	DY10	HaY49	HanY	SaY56	ScY
<u>Spores</u>	-	-	-	-	-	-	-
<u>Ferments</u>							
-glucose	+	-	+	+	+	+	+
-Galactose	-	-	-	-	-	+	-
-altose	-	-	w/D	-	-	+	+
-ellebiose	-	-	-	-	-	+	-
-actose	-	-	-	-	-	-	-
-ucrose	-	-	+	-	-	+	+
-affinose	-	-	+	-	-	+	+
-elibiose	-	-	+w	-	-	+	+
<u>Assimilates</u>							
-glucose	+	-	+	+	+	+	+
-galactose	-	+	+	-	-	+	+
-Sorbose	-	-	+	-	-	-	-
-methyl-D-glucose	+w	D	-	-	-	-	-
-allicin	+	-	-	+	+	-	-
-ebatin	ND	ND	ND	ND	ND	ND	ND
-icrose	-	-	+	+	-	+	+
-ltose	+	-	w/D	+	+	+	+
-libiose	-	-	-	-	-	+	-
-llobiose	+	-	-	+	+	-	-
-ehalose	-	-	-	+	-	+	-
-ctose	-	-	-	+	-	-	-
-ffinose	-	-	+	+	-	+	+
-lezitose	-	-	+	+	-	+	-
-ulin	-	-	+	-	-	-	-

TABLE VIII

Glycerol	+	-	+	+	-	+	-
Erythritol	-	-	+	+	-	-	-
Ribitol	ND	ND	ND	ND	ND	ND	ND
Glactitol	ND	ND	ND	ND	ND	ND	ND
Mannitol	-	+w	+	+	-	-	-
Glucitol	ND	ND	ND	ND	ND	ND	ND
Lactate	-	+	+	-	-	+w	-
Succinate	+w	-	+	O	-	-	-
Nitrate	+w	-	+	O	-	-	-
Nitrate	+	-	+	+	-	-	-
Nitrite	-	-	-	O	O	-	-
Aspartate	+	O	+	+	-	-	-
Asparagine	+	O	+	O	-	-	-
Glutamate	+	O	+	O	-	-	-
Glycine	+	O	O	O	-	-	-
Lysine	+	O	O	O	-	-	-
Nicotin	ND	ND	ND	ND	ND	ND	ND
Glucosamine	+	-	-	-	-	-	-
Erythritol	-	-	+	+	+	-	-
Myo-inositol	ND	ND	ND	ND	ND	ND	ND
α-Me-glucoside	D	-	+	+	-	-	-
Glucose	-	-	-	-	-	-	-
Rows							
30C	+	-	+	+	-	O	+
37C	Q	-	+	-	-	+	-
42C	+	-	+	-	-	+	-
with 50% glucose	ND	ND	ND	ND	ND	ND	ND
Produces glucose	-	-	-	-	-	-	+

nts

[illegible]

TABLE IX

[illegible]

BLE X :

CHARACTERISTICS DISTINGUISHING STRAINS OF YEAST ISOLATED FROM AMARWA

	Ca2 ^a	Ca11	Ha62	Ha32	Sa30M	Sa30N	Sa30P	Sc44M	Sc44N	Sc44p	Sc44T
spores	-	-	-	-	-	-	-	-	-	-	-
ments											
ose	+	+	+	+	+	+	+	+	+	+	+
ctose	-	-	-	+	0	0	+W	-	-	-	-
ose	-	-	-	-	-	-	-	-	-	-	-
ose	+	+	+W	+	0	+W	0	-	-	-	-
ose	-	-	+	+	+	+	+	0	0	0	0
obiose	-	-	-	-	+	+	0	0	+	0	+W
alose	-	-	-	-	+	+	0	0	0	0	0
hilates											
ose	+	+	+	+	+	+	+W	+	+	+	+
ose	+	+W	-	-	+	+	+	+W	+	0	0
ose	+	+	0	0	+	0	0	-	-	-	-
ose	-	-	0	0	0	0	0	0	0	0	0
ctose	-	-	-	-	0	0	0	-	-	-	-
obiose	-	-	-	-	-	-	-	-	-	-	-
alose	-	-	0	0	+	0	0	0	0	0	0
ose	-	-	-	-	-	-	-	-	-	-	-
obiose	-	-	-	+W	+	+W	+W	-	-	-	-
nose	-	-	-	-	-	0	0	0	0	0	0
se	-	-	0	0	0	0	0	-	-	-	-
nose	-	-	-	-	0	0	0	-	-	-	-
nose	-	-	-	-	-	-	-	-	-	-	-
ritol	-	-	-	-	-	-	-	-	-	-	-
tol	+	+	0	0	+	0	0	-	-	-	-
titol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
tol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
roi	+	+	+	+	0	0	0	-	-	-	-
tol	+	+	0	0	+	+W	+W	-	-	-	-
te	-	-	0	0	-	-	-	-	+W	0	0
te	+	+	0	0	-	-	-	0	0	0	0
ate	+	+	0	0	-	-	-	-	-	-	-
ln	-	-	-	-	-	-	-	-	-	-	-
n	-	-	-	-	-	-	-	-	-	-	-
side	+	+	-	-	-	-	-	-	-	-	-
e	-	-	+	+W	-	-	-	-	-	-	-
te	-	-	0	0	-	-	-	-	-	-	-
ic acid	+	+W	0	0	-	-	-	-	-	-	-

TABLE X

at 37°C	+	+	-	-	+	+	0	0	0	0	0
at 42°C	+	+	-	-	+	+	0	0	0	0	0
with 50% Glucose	+	+	-	-	+	+	0	0	0	0	0
with 0.01%	-	-	-	-	-	-	-	-	-	-	-
cycloheximide	-	-	-	-	-	-	-	-	-	-	-
with 0.1%	-	-	-	-	-	-	-	-	-	-	-
cycloheximide	-	-	-	-	-	-	-	-	-	-	-
produces amylose	-	-	-	-	-	-	-	-	-	-	-

See Key to abbreviations

a = Biovars are referred to by the following abbreviations.

Ca = Candida

Ha = Hansunula

SC = Schizosaccharomyces.

ND = Test not done.

0 = Negative response.

- = Not available on this species.

+

TABLE XI

CHARACTERISTICS DISTINGUISHING STRAINS OF YEASTS ISOLATED FROM SORGHUM

	Ca 20	Ca 11	Ha62	Ha32	Sa3OM	Sa3CN	Sa3OP	Sc44M	Sc44N	Sc44P	Sc44t
Ascospores	-	-	-	-	-	-	-	-	-	-	-
Ferments											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Galactose	++	++	++	+	++	+++	++	++	++	++	++
Sorbose	+++	++	++	+	++	+	+	++	++	++	+++
Sucrose	+	+	++	+	W	W	S	+	++	+	+
Maltose	-	+	++	W	++	++	++	+	+	+	++
Cellobiose	++	++	++	++	++	++	+	++	++	++	++
Trehalose	++	+	++	++	+S	S	+	++	++	++	++
Lactose	++	++	++	++	++	++	++	++	++	++	++
Assimilates											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Galactose	++	+	++	+	-	+	-	+	+	-	+
Sorbose	++	++	++	++	++	W	++	++	++	++	++
Sucrose	+	+	++	+	+S	+	+	+	++	+	++
Maltose	+++	+	+	+	-	++	+	++	++	++	++
Cellobiose	+++	++	+	+	++	++	++	++	++	++	++
Trehalose	+	++	++	+	+	S	+	++	++	++	++
Lactose	++	++	++	++	++	++	++	++	++	++	++
Melibiose	++	++	++	++	++	++	++	+	+	++	++
Raffinose	+	+	++	+	++	W	+	+	+	+	+
Mannitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Xylose	++	-	-	+	++	++	++	++	++	++	++
Arabinose	++	++	++	++	++	++	++	++	++	++	++
Rhamnose	++	++	++	++	++	++	++	++	++	++	++
Erythr. tol.	++	++	++	++	++	++	++	++	++	++	++
Ethanol	+	+	++	+	+	++	++	++	++	++	++
Galactitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glucitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glycerol	+	+	++	+	+	++	++	++	++	++	++
Mannitol	+	+	++	++	++	++	++	++	++	++	++
Citrate	++	++	++	+	++	+	++	++	++	++	++
Lactate	+	+S	++	+	W	+	++	++	++	++	++
Succinate	+	+	++	+	-	++	++	++	++	++	++
Salicin	++	++	++	++	++	++	++	++	++	++	++
Starch	++	+	++	++	++	++	++	++	++	++	++
Glucoside	+	+	++	++	++	++	++	++	++	++	++
Nitrate	++	++	++	++	++	++	++	++	++	++	++
Nitrite	+++	++	++	++	++	++	++	++	++	++	++
Aspartic acid	+	+	++	++	++	++	++	++	++	++	++
Glutamic acid	+	+	++	++	++	++	++	++	++	++	++
Glycine	++	++	++	++	++	++	++	++	++	++	++
Lysine	+++	++	++	++	++	++	++	++	++	++	++
Pantothenate	+++	++	++	++	++	++	++	++	++	++	++
Folic acid	+++	++	++	++	++	++	++	++	++	++	++
Niacin	+++	++	++	++	++	++	++	++	++	++	++
Riboflavin	+	+	++	++	++	++	++	++	++	++	++
Thiamine	+++	++	++	++	++	++	++	++	++	++	++
Grows											
W/O Vitamin	++	++	++	++	++	++	++	++	++	++	++
W/O Biotin	+	++	++	++	++	++	++	++	++	++	++
At 30°C	+	+	+	+	+	+	++	++	++	+	+

TABLE XI

At 42°C	-	-	+	+	-	-	-	-	-	-	-
With 50% Glucose	+	+	+	+	-	-	-	-	-	-	-
With 0.01% Cycloheximide-	-	-	-	-	-	-	-	-	-	-	-
With 0.1% Cycloheximide-	-	-	-	-	-	-	-	-	-	-	-
Produces amylose	-	-	-	-	-	-	-	-	-	-	-

* See key to abbreviations

a = Biovars are referred to by the following abbreviations.

Ca = Candida.

Ha = Hansenula

SC = Schnizosaccharomyces.

ND = Test not done.

O = Negative.

- = Not available on this species.

+

W = Weak response.

S = Slow growth

<u>IN JM</u>	a										
i.	Ca2	Ca11	Ha62	Ha32	Sa30	Sa30N	Sa30P	Sc44M	Sc44N	Sc44P	Sc44T

	Ca ⁺⁺	CaCl ₂	NaO ₂	Na ⁺ /2	Ca ⁺⁺	Ca ⁺⁺ /N	Ca ⁺⁺ /r	Se ⁺⁺ /N	Se ⁺⁺ /r	Se ⁺⁺ /r	Se ⁺⁺ /r
Ascospores	-	-	-	-	-	-	-	-	-	-	-
Ferments											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	0	0	0	0
Maltose	-	-	-	-	0	+	0	0	+	+	0
Cellobiose	-	-	-	-	0	0	0	-	-	-	-
Trehalose	-	-	-	-	0	0	0	-	-	-	-

assimilates

[illegible]

Grows

7/0 Vitamin	0	0	0	0	0	0	0	0	0	0
7/0 Biotin	0	0	0	0	0	0	0	0	0	0
At 35°C	-	-	-	0	0	0	+	+	+	+
At 37°C	-	-	-	0	0	0	0	0	+	0

TABLE XII

With 0.01	0	0	0	0	0	0	0	0	0	0	0
Cyclehoximide											
Produces amylose	0	0	0	0	0	0	0	0	0	0	0

^a See Key to abbreviations.

a = Biovars are referred to by the following abbreviation.

b = Candida.

Ha = Hansenula

SC = Schizosaccharomyces

ND = Test not done

0 = Negative response.

- = Not available on this species.

+ = Positive response

W = Weak response

S = Slow growth

PM ii

[illegible]

With 0.01 Cycloheximide	-	-	-	-	-	-	-	-	-	-
With 0.01% Cycloheximide	-	-	-	-	-	-	-	-	-	-
Produces amylose	-	-	-	-	-	-	-	-	-	-

See Key to abbreviations.

- a = Biovars are referred to by the following abbreviations.
- Ca = Candida.
- Ha = Hansenula
- SC = Schizosaccharomyces
- ND = Test not done.
- O = Negative response.
- = Not available on this species.
- +
- = Positive response
- W = Weak response.
- S = Slow growth.

OBSERVATIONS AND DISCUSSIONS

In general, the descriptions of the genera and species were similar to those given by Lodder and Kregger-va-Rij (1980), mainly in the establishment of genera. The monographs were used to identify the following strains of yeasts: Schizosaccharomyces pombe, Saccharomyces cerevisiae, Saccharomyces chevarieli, Saccharomyces pastorianus, Saccharomyces ellipsoides, Debaryomyces hansenii, Dekkera bruxellensis, Lodderomyces elongisporus, Saccharomyces capensis.

The following strains, however, did not confirm fully with the available description on yeasts. The descriptions leading to species deviate from those given by Lodder et al, and are characterised as in Tables II - XII.

Candida Y42, Candida Y50, Candida Y33, Candida 11, Debaryomyces Y46, Debaryomyces Y42, Hansenula Y49, Hansenula Y62, Hansenula 62, Hansenula 32, Saccharomyces Y30, Saccharomyces Y68, Saccharomyces Y20, Saccharomyces Y25, Saccharomyces 12, Saccharomyces 30M, Saccharomyces 30N, Saccharomyces 30P, Schizosaccharomyces Y56, Schizosaccharomyces Y26, Schizosaccharomyces 44M, Schizosaccharomyces 44N, Schizosaccharomyces 44T, Cryptococcus Y57.

The following were yeast strains whose morphological and physiological description did not conform with available descriptions on yeasts, such as those of van der Walt and Yarrow (1984), Kreger van Rij (1984), Phaff and Starnur (1989), Barnett, Payne and Yarrow (1983), to enable the investigator to identify the strains to the genus level. These were coded as follows: Y1, Y2, Y4, Y5, Y7, Y8, Y9, Y10, Y14, Y22, Y28, Y32, Y38 as is shown in table VI.

Because of the above problems, these strains were fully characterised for future identification. Numerical (cluster analysis) has been carried out on some of the above genera. The results were tabulated in Tables II - XIII.

Strains Y1, Y5, Y9, Y10, Y22, and Y32 were highly fermentative strains, Table VI and might belong to the genera that are early fermenters. The early fermenters were identified as Hanseniaspora, Hansenula, Saccharomyces and Schizosaccharomyces. All the strains (Y1, Y5, Y8, Y10, Y22, and Y32) fermented glucose well. This rules out Cryptococcus, Pichia and Rhodotorula species which do not ferment glucose well. The strain Y4 vigorously fermented D-glucose, cellibiose, melibiose and salicin. These characteristics point to Hansenula. However, Y4 did not utilize nitrate. The biovar Y9 had a more similar physiology to Hansenula except that it weakly utilized nitrate.

Y32 compares with *Saccharomyces*. Both produced ascospores, fermented glucose, maltose and sucrose. However, Y32 assimilated galactose, *Saccharomyces* does not assimilate DL-Lactose. This characteristic is absent in Y32. *Saccharomyces* grows well in 2 - 1-glucoside and D-galactose. Both assimilated D-galactose, maltose, sorbose and salicin, which *Schizosaccharomyces* failed to utilize.

Tables II, III, IV summarise the species diversity and population density of yeasts associated with fruits, grains, akiria nuajon, ainyeles, tonto, kwete, ajon/amarwa in Masaka/Rakai, Rukungi, Jinja and Tororo/Mbale regions. The species density differed and diversity varied remarkably in these sources, particularly on surfaces of fruits and grains. No easy explanation can be advanced. However, fruits are known to produce exudates rich in nutrients and these exudates are likely to support yeast flora of varying diversity. Explanation based on factors such as rainfall may not be plausible because these surfaces do not hold water for any reasonable length of time.

However, unlike in Masaka/Rakai where brewing is closely associated with bananas, pawpaws, and passionfruits intercropped with bananas, thus forming an integral part of the shamba, in Tororo/Mbale region the akiria nuajon, ainyeles and ajon/amarwa are prepared on the courtyard. The bananas are not so much associated with brewing processes. The grains, on the other hand, are sun-dried on the same courtyard where ainyeles and akiria nuajon are dried. In Masaka/Rakai, *Drosophila* (fruit flies) bees and butterflies feed on juices during tonto brewing. The same insects have been observed to move and settle on plants in the shamba. Through agency of these insects it is possible for the yeasts to be dispersed to the fruits in the shamba. These hypotheses require further investigation.

INFERTILITY

Isolates, in general, failed to form asci, on all recommended media such as vegetable juice agar, Gorodkova agar, Fowell's acetate agar, Kleyn's acetate agar, McClary's acetate agar, malt extract glucose agar, yeast water agar. Winge and Lausten (1939) in their studies on yeast genetics were the first to prove that the exchange of genetic materials among *Saccharomyces* species was possible when they obtained fertile hybrids for a number of yeast strains which differed in their ability to ferment raffinose.

Winge and Lausten considered that it was justifiable that two types should be recognised as of the same species if their hybrids were fully fertile, but different species if they failed to hybridize or their hybrids showed reduced fertility. Winge and Lausten, Wickerham (1956 a,b) reported the existence of fertility among a group of yeast species. These workers did not attribute infertility as the ultimate criterion in setting the limits of the genus. Guilliermond (1936) raised the possibility that strains of Saccharomyces might be heterothallic, whereby every cell is sexually self-sterile, and requires the aid of another compatible thallus of a different mating type for sexual reproduction.

HETEROTHALLISM

It is only rarely the diplophase and the two sexes of a species which normally exist in the diploid state are isolated simultaneously either from a natural habitat or from an elective (tendency to mate with one sex rather than other) culture, van der Walt (1978).

If only a few or no ascospores are produced the strain may be considered heterothallic.

Ascospore formation (sexual reproduction) among yeasts appears to be a complex process, however, involving nutritional conditions of the culture, pH of the medium, temperature, and degree of aeration, van der Walt (1978). Considerable difficulties were encountered in inducing yeast strains to form ascospores. The investigator made trials on several pre-sporulation and sporulation media but all efforts were futile. Elsewhere we are informed that rusts (Uridinales of the Basidiomycotina) differ in their reproductive cycles. Depending on environmental factors rusts may or may not produce a basidium and basidiospores. The thallus is typically heterothallic, (self-sterile). The thalli occur in approximately equal numbers of sexually incompatible groups. There is evidence that the proportion of micro-cyclic species (where only the teliospore is the only binucleate spore produced) increases with decreasing temperature, Commins (1980). Perhaps the same phenomenon exists among yeasts.

THERMOPHILISM

A thermophilic is defined as an organism that grows above those considered to be the maximum limits for most forms of life, namely between 30C and 80C, such as Bacillus stearothermophilus and Synechococcus eximius. Thermophilism had been suggested by the following observation. It had been observed that hot water was constantly added to the fermenting ajon/amarwa. A few yeasts have been isolated from akiria nuajon after roasting to about 80C. Tonto is brewed under conditions that generate heat to levels that may raise the temperature to over 40C, so much so that, for tonto the banana leaves and grass used to provide heat insulation during fermentation become literally cooked during the process of fermentation. The investigator has recorded temperatures well above 40C during fermentation of tonto. A few yeasts have been isolated from amarwa/ajon and tonto. These are mostly Schizosaccharomyces and Saccharomyces. These yeasts were mostly mesophils. How these yeasts tolerated elevated temperatures during fermentation is a phenomenon that requires thorough study.

NUMERICAL TAXONOMY OF YEASTS
ISOLATED FROM TONTO

BY

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ABSTRACT

During the study a total of 23 strains of yeast were selected and tests were carried out according to Iodder and van der Walt (1984). The results were analysed by computer using Procedure Cluster SPSS/PC+. Of the 72 variables usually used only 60 variables were used to demonstrate degree of similarity among the 23 yeast strains.

When all clusters had been formed at the various levels of similarity, the results were presented in form of a dendrogram which gave hierarchical classification of the yeasts. The complete linkage showed that the 23 yeast strains analysed grouped into 12 clusters at a similarity distance of 10, and 7 at a distance of 14, and 4 of the clusters formed at a distance of 10. Schizosaccharomyces and Hansenula clustered together as expected by conventional methods of identification. The species Debaryomyces (DeY48) was markedly different from all others. It links with HaY17 and HaY4 just below 15. Early fermenters, Sc44M, Sc44N, Sc44T, Sa30N, clustered at a distance of about 10. Rapid growers, CaY42, CaY50 and Ha62 associated below a distance of 10.

INTRODUCTION

Numerical taxonomy aims at a more objective system of classification. This started originally with adoption of the Adamson Principle that all characters of an organism for classification should be given equal weights. As many diagnostic characteristics as possible are used for numerical analysis, and these are formulated as yes (+) or no (-) alternatives.

In conventional identification, a particular yeast is identified by matching the observed cultural, morphological and physiological characters with those recorded in standard monographs such as those of van der Walt and Johannen (1984) for yeasts previously described. A set of 72 or so characters are examined together in order to narrow down the possible classification of an unknown yeast strain.

There are many advantages in having some numerical estimate of the degree of phenetic similarity or difference between a pair of yeasts of which the most obvious is that it can provide a rational basis for deciding the levels of taxonomic rank.

In statistics, the search for relatively homogeneous groups is called Cluster Analysis or Numerical Taxonomy. Normally the term Numerical Taxonomy is applied to systems of classification which are basically taxonomic but in which the degree of similarity of yeast strains, for example, is assessed in quantitative rather than merely qualitative terms, Campbell (1974).

In yeast taxonomy, Cluster Analysis is used to classify yeasts. The aim of Cluster Analysis is to identify homogeneous groups or clusters. The degree of relatedness between yeast strains is a function of the number of similar characters in proportion to the total number of characters examined. The similarities between pairs of strains is then expressed by a similarity coefficient S or some other coefficient that measures distance of similarity. The total number of pairs is given by $\frac{N!}{r!(N-r)!}$.

The concept has been applied to nutritional characteristics of yeast strains, fermentative ability and biomass yield ability.

MATERIALS AND METHODS:

Yeast strains were obtained from yeast stock identified by the conventional method of Lodder et al. Twenty three yeast strains were selected for Procedure Cluster SPSS/PC+.

A total of 60 variables were used. All the variables were expressed in standardized (Z-score) form. Agglomerative hierarchical Cluster analysis, using complete linkage, was used. Distance coefficients were calculated using a programme developed, based on SPSS/PC+. Statistical guide for similarity coefficients. Distance measure was specified by the Cosine of Vectors of variables,

$$\text{Distance } (X,Y) = \frac{(X,Y)}{(\sum X^2)^{1/2} (\sum Y^2)^{1/2}}$$

Once the distance matrix had been calculated vertical and horizontal icecle plots for the 23 yeast strains were made. A dendrogram using complete linkage was drawn.

RESULTS AND DISCUSSION

The identification tests of Lodder & van der Walt (1984) accounted for 60 morphological and physiological characters for numerical taxonomy, Tables XIV-XX. These results were processed by computer SPSS/PC+ to demonstrate distance matching between strains and the results are represented in a vertical and horizontal icecle plots using complete linkage in Tables XXI-XXIII, and in Fig. 1, Fig 2 and a dendrogram in Fig. 3. The 23 clusters at distance 0 show that physiological characteristics alone do not distinguish taxonomic groups of yeasts quite satisfactorily.

The complete linkage dendrogram Fig 3 shows that the 23 yeasts analysed grouped into 6 clusters at a similarity distance of D₄₅ and 4 at a distance of 4 or less. Five of the clusters formed at a distance of 10 contain only one species. These are Sc44T, Sa3OM, DeY48, HaY67, and SaY68. The Species DeY48 was markedly different from all others. It does not link until the distance of about 15. Schizosaccharomyces and Hansenula (Sc44M Sc44N, Sc44T, and HaY49,62, 67) clustered as expected by conventional methods of identification. Otherwise other strains showed varying deviation from the expected association.

Early fermenters, Sc44M, Sc44N Sc44T, Sa3ON, cluster at a distance of about 10, which is expected. Rapid growers, CaY42, CaY50 and Ha62 associated below a distance of 10.

The association of Ha3OP, CaY42 and Sa3OP is rather curious, since Sac3OP and Ha3OP are generally good fermenters. These three strains will require further study to understand why they associate at about 15. A similar situation is observed with Ca11, Ca2 and Sa3OM.

CONCLUSIONS

This study sought to answer a few questions on the feasibility of selecting yeast strains with high ethanol and biomass yield. What yeast strains, and what fermentative characteristics, do the yeasts isolated from tonto may be exploited:-

- a) to yield ethanol and low yield of biomass under anaerobic or batch culture conditions,
- b) to give high biomass yield under aerobic conditions for the purpose of cell production,
- c) to give little change in pH during fermentation,
- d) to increase ability to withstand high levels of ethanol produced during banana juice fermentation should need arise to keep banana wine (tonto) for many years,
- e) for production of large quantities of gas (CO_2).

Taking all these questions into account, 12 strains of yeasts have been selected, using Cluster Analysis to investigate the above characteristics.

The following publications follow from the investigation:-

- a) Influence of Tonto on yeast growth.
- b) Ethanol Tolerance, Ethanol and Biomass yield in yeasts isolated from Tonto.

TABLE XIV :

PHYSIOLOGICAL CHARACTERISTICS FOR NUMERICAL ANALYSIS

1. UTILIZATION OF CARBON COMPOUNDS: FERMENTATION

[illegible]

II. ASSIMILATIVE UTILIZATION OF CARBON HEXOSES (in mg)

<u>STRAIN NO</u>	<u>D-glucose</u>	<u>D-galactose</u>	<u>I-sorbose</u>	<u>Salicin</u>
DeY46	10.8 +	11.2 +	10.6 +	6.4 -
DeY48	10.2 +	6.8 -	14.1 +	13.8 +
CaY42	12.2 +	5.7 -	4.2 -	3.3 -
CaY50	11.7 +	2.5 -	5.3 -	6.7 -
HaY49	15.3 +	4.4 -	6.8 -	11.1 +
HaY62	12.0 +	3.1 -	8.7 -	5.6 -
HaY67	10.1 +	1.2 -	7.1 -	0.1 -
CaY68	13.4 +	6.6 -	6.5 -	6.6 -
CaY56	14.8 +	17.2 +	5.5 -	2.3 -
CaY33	9.9 -	2.2 -	10.9 +	2.1 -
CaY6	7.7 -	8.4 -	5.6 -	3.5 -
ScY56	12.8 +	5.5 -	7.4 -	4.2 -
HaY4	11.3 +	3.2 -	6.7 -	6.2 -
HaY17	14.9 +	3.4 -	9.9 -	4.1 -
HaY68	10.1 +	3.1 -	3.3 -	13.2 -
HaY20	13.1 +	2.2 -	8.7 -	8.1 -
CaY25	12.4 +	15.7 +	5.6 -	6.3 -
CaY12	10.6 +	16.8 +	3.4 -	6.6 -
ScY26	13.7 +	10.1 +	2.2 -	8.7 -
Ca2	17.4 +	8.8 -	17.9 +	6.6 -
Ca11	16.9 +	8.7 -	9.9 -	5.2 -
Ha62	15.3 +	6.4 -	1.2 -	9.8 -
Ha32	10.2 +	6.9 -	8.9 -	3.7 -
Ca30M	12.4 +	5.4 -	10.3 +	1.1 -
Ca30N	11.7 +	6.3 -	4.7 -	9.8 -
Ca30P	9.9 -	5.5 -	5.6 -	9.7 -
Sc44M	10.6 +	9.9 -	6.2 -	4.1 -
Sc44N	12.7 +	14.0 -	3.6 -	5.2 -
Sc44T	15.1 +	8.7 -	6.6 -	9.7 -
Cr57	8.6 -	10.2 -	1.1 -	8.7 -
Ca21	11.8 +	13.2 +	6.2 -	4.5 -

TABLE XVI :

II Contd.

DISACCHARIDES

<u>STRAIN NO.</u>	<u>Sucrose</u>	<u>Maltose</u>	<u>Melibiose</u>	<u>Cellebiose</u>	<u>Trehalose</u>	<u>Lactose</u>
DeY46	10.55	-	10.1 +	12.4 +	6.3 -	6.7 -
DeY48	3.6 -	5.7 -	14.9 +	15.3 +	5.1 -	5.4 -
CaY42	5.5 -	4.5 -	6.1 -	14.8 +	6.4 -	4.3 -
CaY50	2.2 -	3.4 -	5.2 -	8.9 -	6.3 -	7.4 -
HaY49	10.2 +	2.2 -	2.6 -	10.6 +	10.3 +	10.6 +
HaY62	9.9 -	4.4 -	16.2 +	6.6 -	10.4 +	7.5 -
HanY67	8.8 -	6.5 -	4.9 -	5.6 -	14.2 +	8.3 -
SaY30	12.9 +	14.2 +	10.2 +	3.4 -	16.9 +	6.4 -
SaY68	6.9 -	5.5 -	3.9 .	4.7 -	8.9 -	6.5 -
ScY56	11.8 +	2.2 -	2.5 -	3.9 -	6.8 -	5.3 -
CaY33	5.7 -	7.3 -	4.4 -	2.5 -	5.7 -	4.2 -
CaY6	4.5 -	5.4 -	2.7 -	6.4 -	9.8 -	8.4 -
HaY4	15.0 +	3.7 -	8.5 -	12.9 +	10.9 +	6.6 -
Hay17	5.9 -	4.2 -	6.2 -	8.8 -	10.9 +	5.5 -
HanY68	7.3 -	6.6 -	5.3 -	15.4 +	6.5 -	2.5 -
HanY20	12.1 +	5.4 -	6.2 -	14.3 +	3.8 -	1.6 -
SaY21	16.4 +	8.8 -	10.4 +	6.6 -	7.4 -	1.5 -
SaY25	14.3 +	9.9 -	16.3 +	8.3 -	12.1 +	2.3 -
SaY12	16.8 +	14.1 +	15.8 +	7.5 -	16.5 +	6.4 -
ScY26	10.6 +	10.5 +	7.6 -	7.8 -	5.8 -	5.4 -
Ca2	10.9 +	1.2 -	8.3 -	5.2 -	5.4 -	7.3 -
Ca11	14.8 +	7.6 -	9.5 -	4.6 -	5.8 -	6.2 -
Ha62	0.9 -	6.8 -	9.5 -	9.7 -	3.7 -	5.1 -
Ha32	0.8 -	5.7 -	9.9 -	8.2 -	2.2 -	4.6 -
Sa30M	14.7 -	7.2 -	14.8 +	6.1 -	16.8 +	6.5 -
Sa30N	8.6 -	8.9 -	8.9 -	7.1 -	1.4 -	7.7 -
Sa30P	3.7 -	7.5 -	6.9 -	5.6 -	1.3 -	5.3 -
Sc44M	5.6 -	3.8 -	4.3 -	3.2 -	2.9 -	6.4 -
Sc44N	6.4 -	1.2 -	3.5 -	3.9 -	7.9 -	7.5 -
Sc44T	6.8 -	6.2 -	7.6 -	9.8 -	9.4 -	6.6 -
Cr57	8.3 -	7.3 -	10.7 -	8.9 -	8.5 -	6.6 -

TABLE XVII

II. Contd.

TRYSACCHARIDES AND POLYSACCHARIDES

<u>STRAIN No.</u>	<u>Riffinose</u>	<u>Melezitose</u>	<u>inulin</u>	<u>starch</u>
DeY46	11.8 +	13.6 +	0.3 -	12.4 +
DeY48	8.6 -	5.5 -	6.1 -	6.8 -
CaY42	8.9 -	4.3 -	5.3 -	4.1 -
CaY50	10.6 +	3.4 -	3.2 -	5.1 -
HaY49	16.6 +	14.6 +	2.3 -	6.5 -
HaY62	0.8 -	5.5 -	6.4 -	8.3 -
HanY67	14.2 +	8.6 -	0.9 -	6.6 -
SaY30	6.6 -	6.3 -	12.8 +	5.4 -
SaY68	5.7 -	10.9 +	3.9 -	4.2 -
ScY56	4.5 -	5.6 -	4.1 -	3.1 -
CaY33	3.4 -	4.4 -	5.2 -	2.3 -
CaY6	8.5 -	6.5 -	6.1 -	4.2 -
HaY4	6.6 -	8.3 -	7.3 -	6.1 -
HaY17	7.3 -	3.6 -	8.2 -	8.1 -
HanY68	7.7 -	66.6 -	6.2 -	3.2 -
HanY20	6.6 -	7.4 -	5.1 -	5.2 -
SaY21	10.4 +	17.6 +	4.2 -	7.1 -
SaY25	8.9 -	16.4 +	3.6 -	6.2 -
SaY12	17.7 +	16.6 +	0.9 -	9.3 -
ScY26	10.9 +	0.4 -	6.2 -	0.9 -
Ca2	6.6 -	0.2 -	0.4 -	9.1 -
Ca11	5.4 -	0.6 -	0.1 -	2.2 -
Ha62	4.5 -	0.5 -	0.2 -	5.3 -
Ha32	3.3 -	0.4 -	0.3 -	4.1 -
Sa30M	2.7 -	0.3 -	0.1 -	5.1 -
Sa30N	8.8 -	0.2 -	0.1 -	6.1 -
Sa30P	9.9 -	0.6 -	0.2 -	0.3 -
Sc44M	7.4 -	0.7 -	0.1 -	0.4 -
Sc44N	4.6 -	0.8 -	0.3 -	0.8 -
Sc44T	5.7 -	0.5 -	0.6 -	0.6 -
CrY57	6.9 -	10.3 +	0.4 -	0.1 -

TABLE XVIII :

II. Contud.

ALCOHOLS AND ACIDS

STRAIN No.	<u>Ethanol</u>	<u>Glycerol</u>	<u>Mannitol</u>	<u>Lactate</u>	<u>Succinate</u>	<u>Citralin</u>
DeY46	14.1 +	12.3 +	11.0 +	14.2 +	12.7 +	1.9 -
DeY48	10.6 +	6.7 -	4.4 -	13.4 +	14.4 +	4.8 -
CaY42	10.9 +	10.6 +	5.7 -	3.2 -	10.0 +	6.9 -
CaY50	10.2 +	5.2 -	3.1 -	5.3 -	3.2 -	7.5 -
HaY49	16.2 +	4.6 -	11.2 -	3.2 -	2.4 -	6.3 -
HaY62	17.3 +	3.3 -	2.4 -	4.5 -	4.3 -	5.2 -
HanY67	3.6 -	10.8 +	6.6 -	2.4 -	6.2 -	3.8 -
SaY30	5.5 -	5.2	13.6 +	1.3 -	3.6 -	2.5 -
SaY68	4.2 -	4.4 -	8.9 -	4.5 -	2.1 -	4.2 -
ScY56	8.5 -	0.9 -	7.3 -	6.4 -	5.3 -	6.1 -
CaY33	6.4 -	13.4 +	13.7 +	2.6 -	4.2 -	4.3 -
CaY6	7.2 -	14.4 +	12.6 +	6.4 -	6.2 -	4.2 -
HaY4	5.3 -	3.2 -	4.3 -	5.4 -	6.6 -	10.1 +
HaY17	3.2 -	6.4 -	6.8 -	4.2 -	7.4 -	6.1 -
HanY68	2.8 -	8.6 -	3.3 -	3.2 -	8.5 -	8.2 -
HaY20		7.7 -	2.4 -	2.1 -	5.3 -	9.5 -
SaY21	10.3 +	10.5 +	9.9 -	10.6 +	4.4 -	7.6 -
SaY25	10.4 +	14.8 +	5.6 -	10.7 +	6.6 -	7.7 -
SaY12	4.6 -	12.6 +	10.9 +	13.5 +	7.1 -	5.2 -
ScY26	0.7 -	8.4 -	6.5 -	11.2 +	7.6 -	6.4 -
Ca2	10.3 +	10.6 +	14.7 +	15.1 +	10.3 +	16.1 +
Ca11	12.4 +	14.3 +	10.5 +	14.0 +	18.1 +	14.9 +
Ha62	5.3 -	12.4 +	7.6 -	4.3 -	3.2 -	7.2 -
Ha32	3.4 -	10.3 +	6.4 -	6.4 -	4.6 -	8.6 -
Sa30M	12.3 +	7.4 -	15.6 +	5.4 -	3.3 -	9.2 -
Sa30N	6.4 -	5.9 -	5.9 -	2.8 -	5.2 -	6.1 -
Sa30P	8.5 -	6.7 -	4.8 -	4.6 -	8.1 -	5.1 -
Sc44M	7.6 -	7.2 -	5.2 -	3.7 -	8.2 -	4.2 -
Sc44N	6.1 -	6.8 -	6.6 -	2.2 -	7.9 -	5.9 -
Sc44T	4.2 -	8.5 -	6.3 -	1.9 -	2.6 -	3.3 -
CrY57	6.4 -	7.2 -	10.0 +	14.2 +	8.9 -	4.9 -

TABLE XIX

III. UTILIZATION OF NITROEN COMPOUNDS

STRAIN	No. Control (Complete)	Nitrate	Nitrite	Aspart	ArsP	Clut	Crut	Citr.	Org.
DeY46	30.0 +	10.3 +	10.4 +	0.4 -	5.6-	0.6 -	29.1+	30.0+	35.6+
DeY48	34.0 +	8.4 -	10.7 +	0.3 -	6.4-	11.0 +	11.4+	18.6+	20.6+
CaY42	35.0 +	11.5 +	2.8 -	2.1 -	5.0-	9.0 -	15.7+	20.3+	31.4+
CaY50	38.0 +	16.7 +	3.6 -	10.3 +	10.6+	6.0 -	29.8+	25.6+	33.0+
HaY49	40.0 +	10.9 +	5.1 -	17.0 +	10.5+	12.0 +	40.5+	38.5+	29.0+
HaY62	19.0 +	16.3 +	7.1 -	11.0 +	10.3+	6.9-	32.1+	37.7+	20.0+
HanY67	25.0 +	5.2 -	6.4 -	16.0 +	10.4+	18.0 +	22.6+	16.7+	
SaY30	30.4 +	4.1 -	5.2 -	0.2 -	4.1-	7.6 -	32.3+	17.6+	20.0+
SaY68	18.0 +	3.1 -	3.1 -	6.7 -	5.6-	6.9 -	34.6+	19.7+	15.6+
ScY56	18.7 +	10.2 +	2.6 -	6.6 -	3.6-	10.1 +	30.4+	30.0+	37.1+
CaY33	15.4 +	14.6 +	1.1 -	5.0 -	0.7-	0.8 -	0.0-	0.6-	21.6+
CaY6	40.0 +	2.1 -	1.3 -	0.1 -	0.8-	6.0 -	28.1+	23.0+	35.6+
HaY4	36.4 +	10.6 +	5.1 -	0.7 -	0.6-	4.7 -	18.0+	17.6+	30.6+
HaY17	24.0 +	15.7 +	5.2 -	1.2 -	0.2-	5.8-	23.4+	13.6+	20.1+
HanY68	26.0 +	16.8 +	3.3 -	10.0 +	0.3-	8.4 -	10.1+	11.1+	22.6+
HanY20	30.1 +	6.6 -	5.1 -	16.3 +	0.4-	3.3 -	36.8+	17.0+	20.3+
SaY21	38.0 +	6.4 -	6.1 -	17.0 +	0.1-	0.0 -	25.6+	17.6+	34.6+
SaY25	39.2 +	5.2 -	7.1 -	36.6 -	1.5-	4.1 -	11.7+	18.8+	39.5+
SaY12	34.1 +	8.3 -	6.2 -	6.2 -	25.0+	10.1 +	10.3+	24.0+	25.6+
ScY26	39.0 +	9.3 -	1.0 -	6.1 -	0.3-	6.6 -	22.0+	40.0+	30.7+
Ca2	46.0 +	7.1 -	8.2 -	2.3 -	0.4-	3.9 -	23.4+	17.8+	33.3+
Ca11	48.5 +	5.2 -	3.6 -	8.8 -	1.9-	11.0 +	24.8+	30.1+	29.9+
Ha62	28.5 +	16.6 +	2.1 -	6.1 -	0.7-	12.0 +	28.8+	27.0+	34.5+
Ha32	30.4 +	3.9 -	4.1 -	10.2 +	11.0+	15.7 +	33.3+	25.0+	30.4+
Sa30M	36.7 +	2.1 -	5.2 -	11.0 +	12.0+	18.2 +	16.6+	40.0+	36.6+
Sa30N	39.3 +	4.1 -	4.3 -	2.0 -	0.6-	2.0 -	19.9+	29.6+	25.6+
Sa30P	41.1 +	0.1 -	4.1 -	4.0 -	0.4-	11.7 +	20.0+	20.1+	24.1+
Sc44M	37.7 +	0.2 -	5.2 -	0.8 -	0.5-	4.8 -	11.4+	17.7+	20.0+
Sc44N	35.6 +	0.1 -	6.1 -	0.7 -	0.6-	3.7 -	20.0+	16.0+	20.0+
Sc44T	30.4 +	0.1 -	7.1 -	6.0 -	0.3-	4.6 -	29.5+	16.1+	27.4+
CrY57	29.1 +	0.1 -	6.1 -	0.6 -	0.5-	0.6-	20.1+	18.6+	14.7+

TABLE XX :

IV. GROWTH: in Vitamin - free medium; on 50%(w/w) glucose-yeast extract agar; at 30°C, 37°C, 42°C.

<u>STRAIN No.</u>	<u>V.f(w/o)</u>	<u>50% glucose</u>	<u>30C</u>	<u>37C</u>	<u>42C</u>
DebY46	6.3 -	6.4 -	10.6 +	0.6 -	0.0 -
DebY48	12.4 +	3.1 -	12.8 +	3.1 -	0.2 -
CaY42	10.2 +	1.2 -	41.9 +	10.2 +	0.0 -
CaY50	10.8 +	1.1 -	40.6 +	10.6 +	0.0 -
HaY49	7.6 -	3.2 -	16.7 +	14.0 +	0.1 -
HaY62	16.7 +	2.0 -	17.8 +	16.0 +	0.1 -
HanY67	2.1 -	3.1 -	26.7 +	8.6 -	0.1 -
SaY30	4.2 -	2.3 -	20.4 +	6.7 -	0.3 -
SaY68	5.3 -	4.6 -	29.6 +	10.6 +	0.4 -
ScY56	3.6 -	1.1 -	30.4 +	5.6 -	0.5 -
CaY33	2.1 -	1.2 -	28.3 +	0.3 -	0.1 -
HaY4	4.3 -	6.9 -	12.0 +	6.6 -	0.4 -
HaY17	2.1 -	7.7 -	10.1 +	7.8 -	0.0 -
HanY68	1.1 -	1.8 -	10.3 +	8.1 -	0.0 -
HanY20	2.1 -	7.7 -	10.6 +	0.9 -	0.0 -
SaY21	5.1 -	2.4 -	40.2 +	0.8 -	0.0 -
SaY25	3.3 -	6.6 -	38.4 +	0.6 -	0.0 -
SaY12	3.2 -	7.8 -	34.9 +	0.7 -	0.0 -
ScY26	6.1 -	2.1 -	20.7 +	9.9 -	0.0 -
Ca2	1.7 -	2.6 -	21.5 +	8.6 -	0.0 -
Ca11	2.3 -	4.9 -	23.6 +	5.7 -	0.0 -
Ha62	6.7 -	3.1 -	40.7 +	9.9 -	0.0 -
Ha32	3.6 -	2.1 -	23.3 +	8.1 -	0.0 -
Sa40M	2.7 -	3.0 -	29.7 +	9.3 -	0.0 -
Sa30N	3.8 -	2.1 -	30.6 +	6.5 -	0.0 -
Sa30P	5.6 -	6.6 -	33.4 +	7.2 -	0.0 -
Sc44M	6.6 -	7.7 -	29.9 +	2.7 -	0.0 -
Sc44N	3.7 -	6.2 -	28.7 +	9.9 -	0.0 -
Sc44T	4.4 -	6.0 -	20.1 +	8.9 -	0.0 -
Cr57	7.2 -	7.6 -	14.5 +	1.3 -	0.0 -

Data Information

24 unweighted cases accepted.
 12 cases rejected because of missing value.

Cosine measure used.

Table XXI

1 Agglomeration method specified.

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Agglomeration Schedule using Complete Linkage

9/16/93

Stage	Cluster 1	Cluster 2	Coefficient	Stage Cluster 1	1st Appears Cluster 2	Next Stage
1	22	23	.754652	0	0	11
2	1	2	.639485	0	0	14
3	19	20	.557675	0	0	7
4	4	5	.513173	0	0	8
5	8	18	.445445	0	0	15
6	12	13	.405114	0	0	16
7	6	19	.385364	0	3	19
8	4	9	.372703	4	0	15
9	3	11	.330766	0	0	18
10	14	15	.324277	0	0	17
11	22	24	.305065	1	0	13
12	10	21	.249758	0	0	17
13	17	22	.225075	0	11	18
14	1	16	.222620	2	0	19
15	4	8	.086030	8	5	21
16	7	12	.031952	0	6	20
17	10	14	-.010638	12	10	20
18	3	17	-.101648	9	13	21
19	1	6	-.164051	14	7	22
20	7	10	-.164517	16	17	22
21	3	4	-.261571	18	15	23
22	1	7	-.467494	19	20	23
23	1	3	-.558659	22	21	0

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9/16/93

Horizontal icicle plot Using Complete Linkage

Number of Clusters

-111-

Table XXII Hierarchical Cluster Analysis

Number of Valid Observations (Listwise) =

24.00

Variable	Mean	Std Dev	Minimum	Maximum	N	Label
D_GLUCOS	12.25	2.35	7.7	17.4	31	
D_GALACT	7.53	4.42	1.2	17.2	31	
L_SORBOS	6.93	3.61	1.1	17.9	31	
SALICIN	6.35	3.33	.1	13.8	31	
SUCROSE	8.79	4.50	.8	16.8	31	
MALTOSE	6.43	3.26	1.2	14.2	31	
MELIBIOS	8.19	4.12	2.5	16.3	31	
CELLEBIO	7.84	3.97	.1	15.4	31	
TREHALOS	7.95	4.26	1.3	16.9	31	
LACTOSE	5.89	2.02	1.5	10.6	31	
RAFFINOS	7.81	3.80	.8	17.7	31	
MELEZITO	5.85	5.47	.2	17.6	31	
INULIN	3.26	3.21	.1	12.8	31	
STARCH	4.82	3.01	.1	12.4	31	
ETHANOL	7.83	4.08	.7	17.3	30	
GLYCEROL	8.43	3.62	.9	14.8	31	
MANNITOL	7.86	3.71	2.4	15.6	31	
LACTATE	6.55	4.47	1.3	15.1	31	
SUCCINAT	6.40	3.36	2.1	18.1	31	
CITRALIN	6.58	3.11	1.9	16.1	31	
CONTROL	32.70	8.16	15.4	48.5	31	
NITRATE	7.48	5.51	.1	16.8	31	
NITRITE	4.99	2.37	1.0	10.7	31	
ASPART	6.81	6.37	.1	25.0	31	
ARSP	4.11	4.42	.1	12.0	31	
GLUT	7.31	5.46	.0	20.0	31	
ORNIT	23.70	8.95	.7	40.5	31	
CITR	22.96	9.29	.6	40.0	31	
ORG	27.43	7.39	14.7	39.5	30	
VFWO	5.23	1.51	1.1	16.7	30	
COGLUCO	4.14	3.42	1.1	7.8	30	
COO	24.97	10.16	10.1	41.9	30	
COO	6.66	4.22	.3	16.0	30	
COO	.07	.14	.0	.5	30	

Table XXIII Hierarchical Cluster Analysis

From Variable	To Z-score	Weighted Valid N
D_GLUCOS	ZD_GLUCC	31
D_GALACT	ZD_GALAC	31
I_SORBOS	ZI_SORBO	31
SALICIN	ZSALICIN	31
SUCROSE	ZSUCROSE	31
MALTOSE	ZMALTOSE	31
MELIBIOS	ZMELIBIO	31
CELLEBIO	ZCELLEBI	31
TREHALOS	ZTREHALO	31
LACTOSE	ZLACTOSE	31
RAFFINOS	ZRAFFINO	31
MELEZITO	ZMELEZIT	31
INULIN	ZINULIN	31
STARCH	ZSTARCH	31
ETHANOL	ZETHANOL	30
GLYCEROL	ZGLYCERO	31
MANNITOL	ZMANNITO	31
LACTATE	ZLACTATE	31
SUCCINAT	ZSUCCINA	31
CITRALIN	ZCITRALI	31
CONTROL	ZCONTROL	31
NITRATE	ZNITRATE	31
NITRITE	ZNITRITE	31
ASPART	ZASPART	31
ARSP	ZARSP	31
GLUT	ZGLUT	31
ORNIT	ZORNIT	31
CITR	ZCITR	31
ORG	ZORG	31
VFWO	ZVFWO	30
@OGLUCO	Z@OGLUCO	30
@OC	Z@OC	30
@7C	Z@7C	30
@2C	Z@2C	30

C A S E 12345678901234567890123
 Label Seq ++++++

-113-

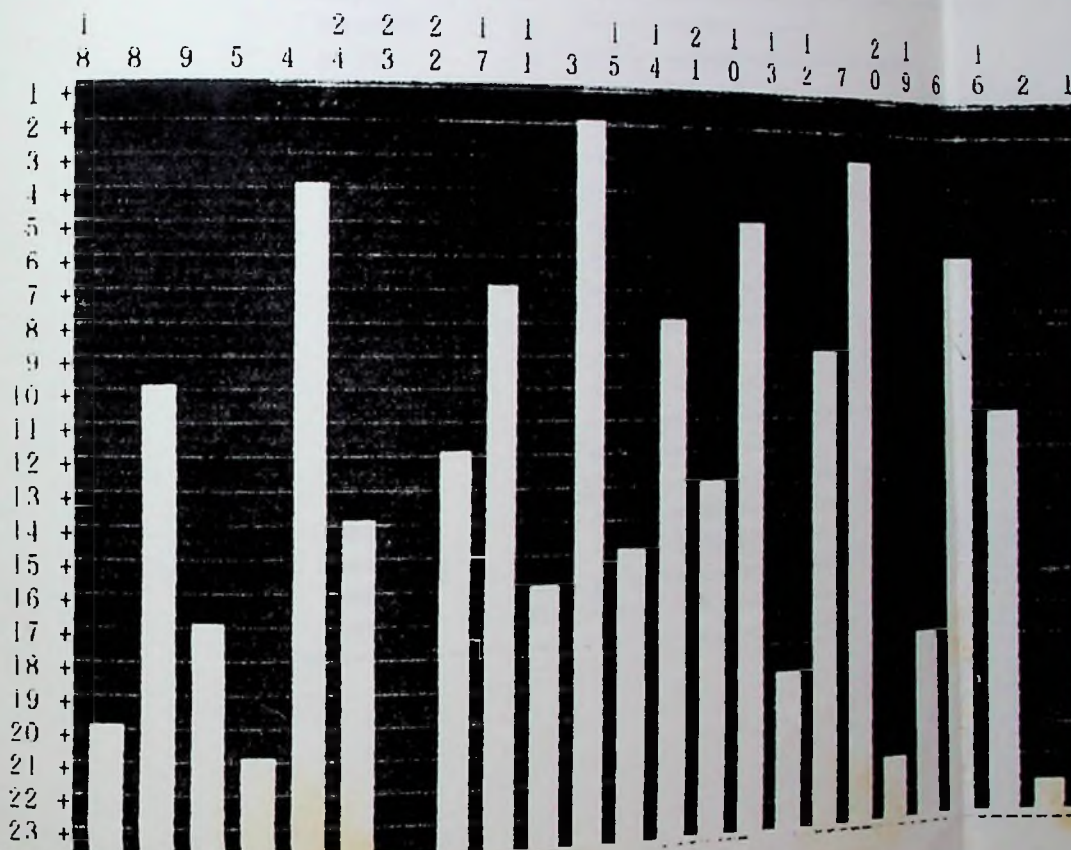
Fig 8

sa30p	18	
ha32	8	
ha62	9	
cay50	5	
cay42	4	
sc44t	24	
sc44n	23	
sc44a	22	
sa30n	17	
hany68	11	
cay33	3	
hay62	15	
hay49	14	
say68	21	
hany67	10	
hay4	13	
hay17	12	
dey48	7	
say25	20	
any12	19	

Vertical Ice Plot using Complete Linkage

(Down) Number of Clusters (Across) Case Label and number

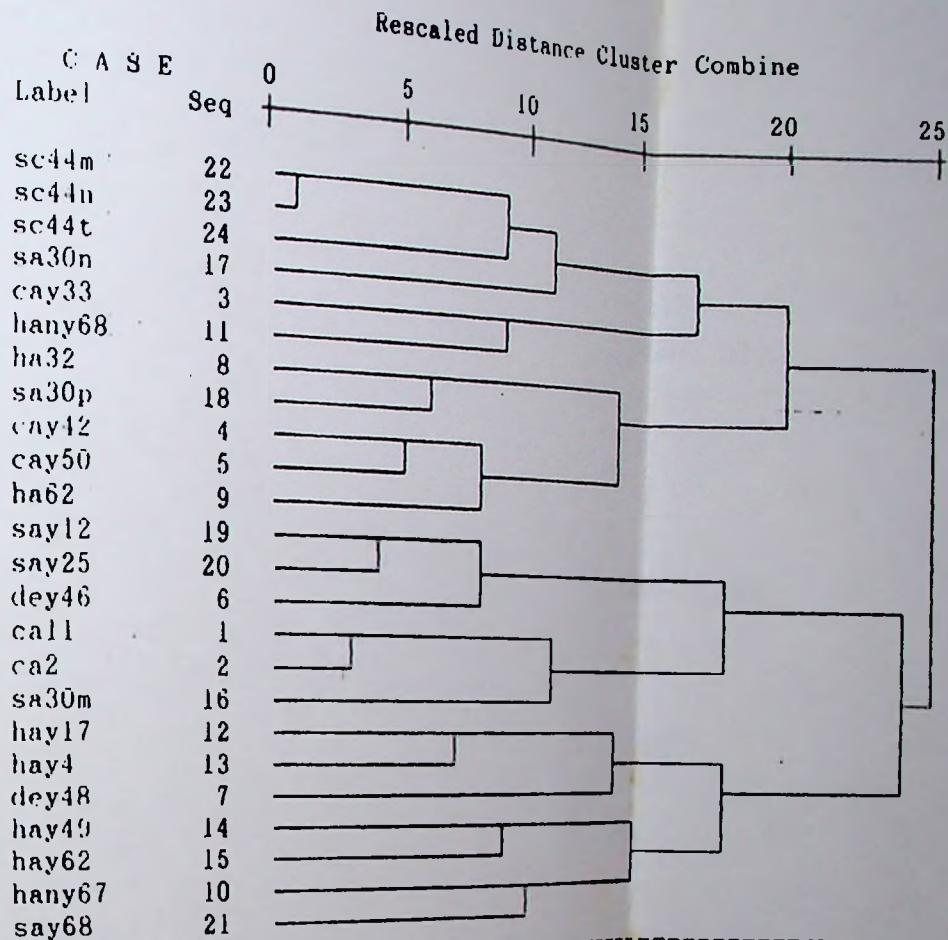
s	h	h	c	c	s	s	s	s	h	c	h	h	s	h	h	h	d	s	s	d	s	c	c
a	a	a	a	a	c	c	c	a	a	a	a	a	a	a	a	a	e	a	a	e	a	a	a
3	3	6	y	y	4	4	4	3	n	y	y	y	y	n	y	y	y	y	y	y	3	2	1
0	2	2	5	4	4	4	4	0	y	3	2	9	8	6	4	1	7	8	5	2	6	0	1
p			0	2	t	n	m	n	6	3	2			7							m		



SPSS/PC+

Dendrogram using Complete Linkage

Fig 10



APPROXIMATE CHEMICAL ANALYSIS OF TONTO

BY

KAFEERO KIWANUKA COSMAS

ABSTRACT

Under traditional brewing techniques tonto deteriorates rapidly. Only after 48 hours the brew is sour, Kafeero (1994): Influence of tonto on yeast growth, together with pilot studies. The brew is unpalatable due to rapid exhaustion of sugars and accumulation of acids and phenolic compounds.

The study sought to answer questions on the feasibility of producing tonto as a wine, commercially sustainable in terms of quality.

The results show that it is possible today to produce tonto that compares well with grape or apple wines produced in Europe or elsewhere in the industrialized countries on the basis of the experience acquired from this study.

From the results in Table.... starting with a high concentration of sugar of 20%, at the end of 5 weeks the tonto is still sweet, with about 8% sugar still remaining. Fermentation during the same period yield was 20.99%. There was reasonably little change in pH over the same period. Hardly any phenolic compounds accumulated during the period. In fact, at the end of 5 weeks, the wine was "good": sweet, alcoholic and with good flavour, as observed by one with a good taste for wines.

METHODOLOGY

The tonto was prepared as usual (method described earlier), except that pure strains of yeasts were used and strict asepsis was observed. The strains were the 12 used before in the previous experiments.

Analysis:

1. Sugar: Reading Brix....by using the refractometer and then converting to percentage sugar using Tables (A,C,A,C).
2. Alcohol: Taking the specific gravity using the specific gravity bottles and converting to percentage ethanol from Tables (A,O,A,C).
3. Calcium: Perchloric acid...determined titrametrically using potassium permanganate digestion.

5. Iron: Ref off from the Atomic Absorptiometer, recorded in at X.

RESULTS: The results were recorded in Table....

DISCUSSION

There are no other techniques required to improve on the quality of tonto. The results of this investigation show that what is required is an initial high sugar content from bananas, that is no water should be added to the juice extract from properly ripened fruits, especially Kisubi, Kayinja, bogoya and sukari, used in combination. These observations follow from Pasteur Effect. In these circumstances, growth is virtually anaerobic and the sugars assimilated are metabolised to CO_2 and ethanol by the process of fermentation, until the sugar concentration has been reduced to a very low level of about 0.1%. When the sugar concentration has been reduced to very low levels, the ethanol is re-utilized and is oxidised to CO_2 and water, producing phenolic compounds. The wine then tastes "bad". Presence of Lactobacter and Acetobacter further leads to wine (tonto) spoilage by converting the ethanol to lactic and acetic acids. The tonto becomes sour.

It is, therefore imperative that the containers are kept as clean as possible to avoid heavy inocula of the bacteria.

If possible, selected yeast strains should be used to maintain good standards of banana wine quality.

TABLE XXIII APPROXIMATE CHEMICAL ANALYSIS OF TONTO

ANALYSIS	DATES OF SAMPLING					
Sample	0	1	2	3	4	5
	23/8	24/8	25/8	26/8	2 weeks	5 weeks
Sugar %	20	19.75	19.5	9.5	9.5	8.0
Alcohol %	4.39	5.39	6.44	9.3	12.9	20.99
pH	4.8	5.02	-	5.03	4.7	3.75
Acidity Acetic acid % (W/V)	-	0.29	-	0.33	0.36	1.26
Calcium mg/ 100	3	9	1.8	-	2.8	5.0
Phosphorus ppm	-	0.03	0.019	-	0.025	0.026
Iron ppm	0.5	0.5	0.1	0.1	-	35
Protein	0.1	0.13	0.17	-	-	-

INFLUENCE OF TONTO ON YEAST GROWTH

BY

KAFETRO KIWANUKA COSMAS

DEPARTMENT OF BOTANY MAKERERE UNIVERSITY

ABSTRACT

TONTO is a sweetish, semifermented banana² juice mixed with roasted and ground sorghum (Sorghum bicolor) to give colour and flavour to the beverage. The bananas are a variety that is bitter when steamed and, therefore, not good for the traditional Kiganda dish called matooke. The beer bananas are of the Musa sapiens varieties called mbidde (Ganda) with genomes AAA, AAB and ABB triploids.

During the study a total of 12 yeast strains were selected. In this study, stimulation and inhibition, growth, and killing of yeast cells during the process of fermentation were investigated. Killing and death in yeasts were defined as the irreversible loss of the ability to grow and reproduce as it related to experimental conditions. The term "killing"/"death" related to the loss of colony forming ability.

Quantitative estimations of killing and death in yeasts isolated from Tonto were measured for populations and not for individual cells. It was observed that in many cases the rate of loss of viable cells at any one time was proportional to the first order reaction kinetics.

$$N = N_0 e^{-kt} \quad (k = \text{the exponential death rate}).$$

The killing rate was dependant on a number of factors in the fermenting banana juice.

Some of the factors included phenolic compounds, pH change and accumulation of ethanol and depletion of glucose.

REPRODUCTION

Searchers on physiology of microorganisms have come up with several meanings for the word growth. Growth, as a metabolic process may be considered as the irreversible increase in living substance in living organisms, by the synthesis of macromolecules. Living organisms can grow by increase in mass sometimes by cell division and an increase in cell numbers.

In fission of budding yeasts increase in number is by growing until almost double their original size and then dividing into two. If environmental factors (temperature, nutrients, pH, etc..) are suitable the process is repeated so that all the yeast cells continue to grow and divide.

Yeasts reproduce either by fission or budding. Their multiplication, therefore, corresponds to a geometric progression.

$$2^0 \rightarrow 2^1 \rightarrow 2^2 \dots \dots \dots 2^n$$

This process enables one cell to become successively, 1, 2, 4etc... with no mathematical limit. Using simplifying assumptions that if cell divisions occur at equal time (t) intervals and all cells divide, with the same rates, and that none are dying, then the logarithm of cell number plotted against time will yield a straight line, giving the expression logarithmic growth.

$$\log N = \log N_0 + n \log 2 \text{ (a straight line).}$$

However, such situation as described above rarely exists in growth. If the total mass measurements are made on a culture of yeast cells which are growing, the base for logarithms is the exponential (e), and the cells are found to be growing exponentially. The values for the exponential function describes a characteristic curve given by

$$y = ba^t$$

There are two types. The growing exponential function when $a > 1$; and when $a < 1$ it is said to be a death exponential function.

After a given time of growth, growth begins to decrease. If t is the time in hours, after maximum growth is reached it appears that an exponential death of the type

$$y = ba^t$$

describes the situation, where a and b are constants to be determined from experimental data, and $a < 1$.

All death in yeasts may be defined as the irreversible loss of the ability of the cells to form colonies on a suitable medium. In many cases the rate of loss of viable cells at any one time has been found to be proportional to the number of viable cells. It therefore, seems reasonable to assume that death of cells follows first order reaction kinetics,

$$N = N_0 e^{-kt}$$

The killing and death of yeasts are fundamental in brewing and baking and production of biomass and pharmaceuticals. The process is also important in food preservation. Growth and death of yeasts will determine the amount of ethanol and biomass the cells will produce and whether they can withstand or tolerate the environment during the process of fermentation of metabolism.

Initially Tonto provides an ideal medium for yeast growth. Tonto is a sweetish medium, containing hexoses about 200g/l. It contains amino acids and proteins, including a variety of mineral salts, such as calcium, phosphorus and a variety of vitamins.

MATERIALS, EQUIPMENT AND METHODS

Medium

Banana juice well mixed with roasted sorghum (S.bicolor).

Materials : six ripe clusters of each of Kisubi, Sukari, Kayinja (All Musa varieties genome ABB); 20 kg of ripe fingers of each variety, were used for juice extraction; grass (Imperataspp) was used during squeezing or extraction of the juice, 5kg of roasted and ground sorghum were used.

Equipment: Two large, clean sauce pans, 20 litre jerry cans, 500 ml
sieves.

Labour: Two ladies were employed to squeeze juice from the ripe
bananas; to roast and grind the sorghum.

Method: The ripe bananas with skins on were mixed with the grass in a
large (20 litre sauce pan); juice was manually extracted out of the
ripe bananas using grass to provide fibre; roasted and ground sorghum
was added and the total extract of 40 undiluted fresh tonto was put
into 2 jerrycans and the tonto allowed to ferment. The yeast flora
naturally occurs on the banana fruits, as wild yeasts. The juice was
allowed to ferment for 5 weeks at 23°C.

The aim of the above procedure was to simulate the traditional way of
brewing tonto.

Yeasts were isolated, yeast number and biomass were recorded, approx-
imate chemical analysis was done and results recorded. The para-
meters of the analysis included crude hexoses, proteins, amino acids,
calcium, phosphate iron, ethanol, acetic acid and lactic acid and
phenolic compounds. All the recordings were done at 0, 24, 48, 72, 96,
120, 144, 168, 2 weeks, 5 weeks, on samples taken at these time intervals.

Total cell count was determined by enumeration using a haemocytometer.
Viable counts (colony forming units, c.f.u) involved counting the
colonies produced by the viable cells on nutrient agar. Biomass was
determined after centrifuging and washing with distilled water and
drying at 100°C to constant weight in an oven, and the dry weight
determined using an electronic balance. Dry weight was taken after
1, 2, 3, 4 and 5 weeks. It was appreciated that this procedure was
subject to systematic errors. However, these were the best means at
hand. The errors originated from residual medium and drying. The
errors were corrected using simplifying assumptions of an error not to
exceed 2%. Hence, the error was given by $100 \times dx/x-2$ where x is the
mass measured in mg. The method was essentially batch culture.

RESULTS AND DISCUSSION

The results are given in Figure 11-12.

After 5 weeks the tonto contained less than 0, 1% crude hexoses... protein 0 amino acids 0 calcium 5mg/100 phosphate 0.03 ppm iron 35 ppm ethanol, total acid 0.80% and pH 5.3. The methods used for these analyses are those described in the Appendices.

The results in Figure 11 give the situation after 20 days of growth and fermentation.

The growth patterns of yeasts, c.f.u. are plotted in Fig. 11. Colony forming units rise from about 16 to more than 60 after 40 hours. Thereafter the c.f.u decline to about 8 after 480 hours. Meantime, ethanol increases sharply from 0 to over 18g/l within 24 hours of fermentation. Thereafter ethanol decreased to about 1.2g/l after 480. The rapid rise in ethanol is paralleled with a sharp fall in sugar concentration, so that after only 40 hours the sugar level is less than 0lg/l. Fig. 12 shows percentage survivors of yeasts. There is a rapid, exponential, death of yeast cells after 40 hours of fermentation. The cells are nearly all killed by 120 hours of fermentation.

Batch culture methods rely on altering the overall nutritional composition of the medium or by changing the sugar source. The investigation starts with banana juice rich in hexoses, mineral salts and proteins, with no ethanol or phenolic compounds. In these circumstances, growth is virtually anaerobic and the sugars assimilated are metabolised to CO_2 and ethanol by the process of fermentation, until the sugar concentration has been reduced to a very low level. When the sugar concentration has been reduced to very low levels, the ethanol is re-utilized and is oxidised to CO_2 and water, producing phenolic compounds. Under these circumstances yeast cells start dying rapidly, according to the first order of reaction kinetics,

$$N = N_0 e^{-kt}$$

The killing rate was dependent on a number of factors in the fermenting banana juice. Some of the factors included depletion of nutrients and accumulation of toxic phenolic compounds.

Since the investigation was a simulation of the traditional method of tonto brewing, the technique did not take aseptic precautions. Much of the acid produced and degradation of ethanol might have involved lactic acid and acetic acids bacteria.

CONCLUSIONS

Under traditional brewing techniques tonto deteriorates rapidly. Only after 48 hours the brew is sour and unpalatable due to the rapid exhaustion of sugars and accumulation of acids and phenolic compounds.

This study sought to answer a few questions on the feasibility of producing tonto as a wine, commercially sustainable in terms of quality and quantity.

1. Is it possible today to produce tonto that compares with grape wines produced in Europe or elsewhere in the industrialized countries on the basis of the experience acquired from this study and traditional methods?

Should other techniques be introduced to improve on the quality of tonto?

2. What quality of banana wine is possible given proper conditions of brewing, such control of prokaryotic contamination or higher levels of sugar or both?

It is a well known fact that with high level of sugar concentrations yeasts shift their metabolism from oxidation of ethanol fermentation, the Pasteur Effect. This effect can be applied in TONTO brewing, as a wine.

3. What yeast strains and what fermentation characteristics do these yeasts isolated from TONTO may be exploited:

- a) to yield ethanol and low yield of biomass under batch conditions,
- b) to give high biomass yield under aerobic conditions for the purpose of cell production,
- c) for production of large quantities of gas (CO_2); for effective flocculation ability to aid in yeast harvesting?

Taking all these questions into account, 12 strains of yeast have been selected, using Procedure Cluster Analysis, to investigate association among the yeasts for ethanol production and biomass (growth) yield in yeast isolated from TONTO.

YEAST GROWTH IN BATCH CULTURE

125

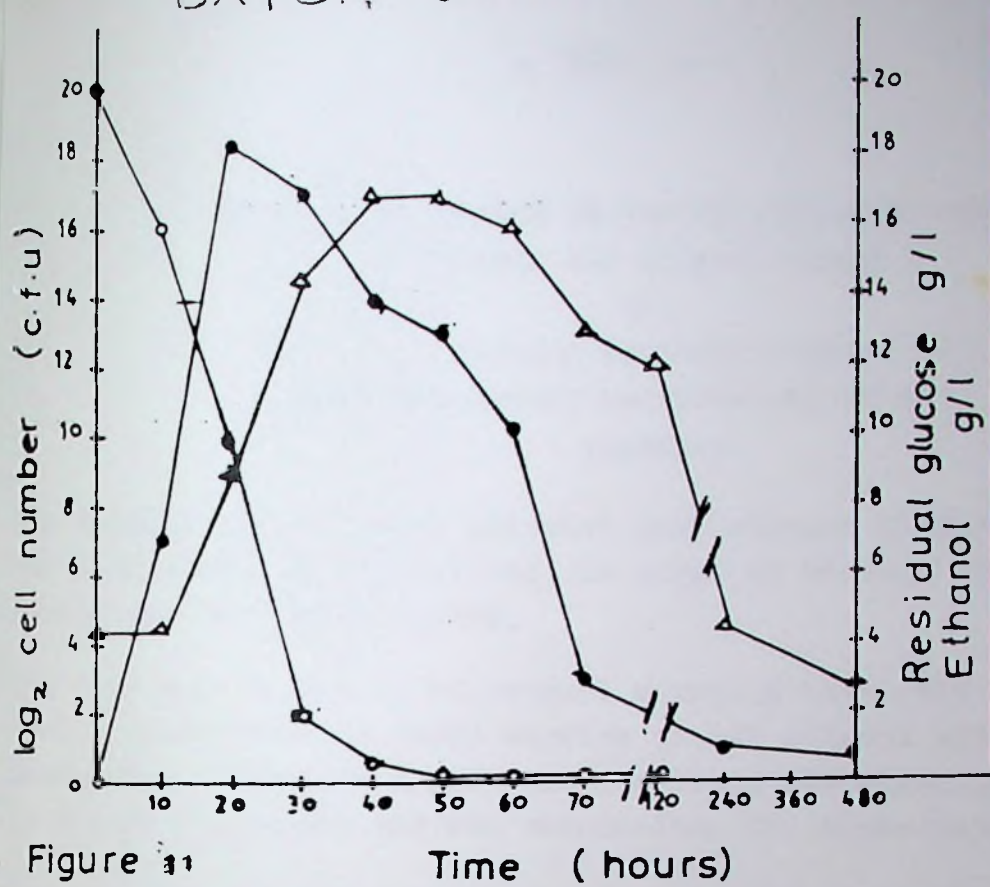


Figure 11

- △ c.f.u
- residual glucose
- ethanol

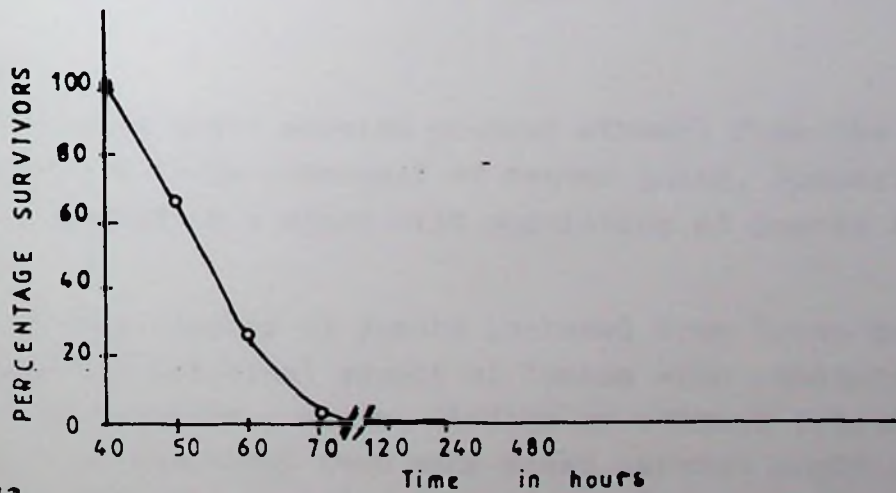


Fig 12

ETHANOL TOLERANCE IN YEASTS ISOLATED FROM TONTO,
ETHANOL AND BIOMASS YIELD

BY

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ABSTRACT

The feasibility of using selected pure strains of yeasts from TONTO for high yield of ethanol and low yield of biomass under batch culture conditions was investigated.

The test for tolerance of ethanol revealed that only 6 out of the twelve yeast strains could survive at 14% ethanol after 72 hours of incubation. These 6 types show more than 50% viability after 3 weeks in fermenting tonto and was responsible for tonto maturing.

Values obtained for ethanol yields and biomass yields in the batch culture fermentation demonstrated that most of the yeasts fermented glucose to yield ethanol. It was noted that, in relative terms, most of the sugar was utilized to produce ethanol rather than for biomass production, with the exception of the strains Ca2 and Ca11.

INTRODUCTION

The twelve selected yeast strains produce ethanol from the fermentation of glucose, a major component of banana juice, Purseglove (1991). Tonto brewers depend on a mixed wild population of yeasts for the fermentation.

This aspect of the biology of yeasts isolated from Tonto has not been studied beyond the practical aspect of banana wine production by a few adventurous Ugandans. During studies on ethanol tolerance of the twelve yeasts, it was noted that many yeast strains could not survive at 14% ethanol for long periods of storage. This is often the problem experienced by cottage tonto brewers in Uganda.

Thus at the outset of the study it was thought that high ethanol yields and tolerance by yeast cells might be useful characteristics to the prospective banana wine producer.

MATERIALS AND METHODS

Strains (yeasts) were strains isolated from TONTO during previous phase as described earlier.

Strains were SaY30, SaY21, SaY25, HanY68, Sc44, DeY46, DeY48, 17, ScY26, Ca2, Cal1 and HanY62.

Media Glucose peptone agar (GPA) contained 2% (w/v) Bacto Peptone and 2% (w/v) Bacto Agar. Glucose peptone broth (GPB) had the same constituents without agar. Yeast Nitrogen Base without amino acids (YNB) was prepared in accordance with the manufacturers' instructions (See Appendix), Difco Laboratories of Detroit (Michigan).

Methods:

Ethanol tolerance: Cells of yeast strains were grown in 50 ml YNB with 1% glucose contained in 300 ml nepheloflasks with side arms to control evaporation. Ethanol of at concentrations of 1,2,5,10,14% (v/v) was added to the flasks at the time of inoculation. Cells for study were grown on GPA at 23°C for 24 hours, harvested and washed twice in sterile water. Growth was initiated at a cell concentration of about 5×10^6 cells ml⁻¹ (by serial dilution). The cultures were grown in three litre sterile containers at 23°C and growth was assessed at regular intervals as follows:

Growth was measured by direct method of dry weight. After drying to constant weight at 100°C in an oven, dry weight was determined by weighing on an electron balance.

Determination of ethanol yield by the selected yeast strains: Cells of the selected strains were grown in yeast extract peptone broth, for ethanol fermentation, with 2% glucose at 23°C. The level of ethanol was determined using an alcoholimeter over a period of 72 hours.

RESULTS

Table XXV. Ethanol Tolerance (growth in mg after 72 hours)

Ethanol (%)	1%	2%	5%	10%	14%
Strain					
Y46	14.6	14.2	10.4	8.3	5.1
Y48	11.0	7.3	7.0	6.4	5.0
Y21	10.8	10.0	10.0	8.7	8.0
Y25	8.0	6.0	3.0	0	0
44M	6.4	4.2	2.5	1.7	1.0
Y68	3.0	1.0	0	0	0
Y17	3.4	.09	0	0	0
Y26	0.7	0	0	0	0
Y30	.6	5.0	3.1	0	0
Y62	12.0	10.1	10.0	.0	8.2
2	10.7	9.8	8.0	8.0	7.6
all	13.5	12.3	12.0	12.0	11.1

Table XXVI. Ethanol and biomass yield (after 72 hours)

Strain	Ethanol yield (%)	Biomass yield (mg)
Y46	3.1	0.2
Y48	2.2	0.4
Y21	4.3	0
Y25	4.1	1.2
44M	3.4	1.7
Y68	1.0	1.0
Y17	1.0	0.4
Y26	1.1	3
Y30	4.1	1
Y62	3.3	9
2	1.2	12.8
all	1.3	14.1

RESULTS AND DISCUSSION

Twelve strains of yeasts were inoculated in triplicate into flasks of Yeast Nitrogen Base with 1, 2, 5, 10 and 14% ethanol and the results are recorded in Table..... Values obtained for ethanol tolerance revealed that only 6 of the 12 yeast types could survive at 14% ethanol concentration. These included DeY46, DeY48, SaY21, Ca2, Call and HaY62. The others, SaY25, Sc44M, HanY68, HaY17, ScY26 and SaY30 hardly survived 5% ethanol concentration.

Production of ethanol and biomass on glucose 20g/l cultured for 72 hours. Values obtained for ethanol yield and biomass yield are recorded in Table..... The results demonstrate that most of the yeasts fermented glucose. The mean ethanol yield was 2.5% with standard deviation of 1.34%. Biomass yield had a mean weight of 3.7 mg and standard deviation of 5.2 mg. There was relatively more variability in biomass yield among the 12 selected yeast strains than in ethanol yield.

Storage of fermented Tonto is a problem to the cottage tonto brewer in Uganda. Tolerance of ethanol among tonto yeasts is, therefore, an important characteristic in brewing industry. Stimulation or inhibition of growth or killing or death of yeast cells in tonto were investigated. Killing and death in yeasts were defined as the irreversible loss of ability to grow and reproduce as it related to experimental conditions outlined in the methodology. The term killing or death related to the loss of colony forming ability. Quantitative estimation of growth, killing and death in yeasts isolated from tonto were measured. The killing rate was dependent on the ethanol concentration in the fermenting medium. The results show that strains DeY46, DeY48, SaY21, Ca2, Call and HaY62 were highly tolerant of ethanol in comparison to strains SaY25, Sc44M, HanY68, HaY16, ScY26, and SaY30. These could be said to be poorly tolerant by the standards of Casey & Engledow (1936).

was observed that 5% ethanol wiped out HaY68, HaY17 and ScY26. Growth of Sc44M was reduced by more than 50% at 5% ethanol concentration. Ethanol tolerance might be genetically controlled. This needs to be investigated by controlled crosses and selection.

The production of ethanol and biomass by 12 selected yeasts was investigated and the results recorded in Table..... The mean ethanol yield was 2.5% and standard deviation 1.34% in 72 hours. The values for biomass were a mean yield of 3.7mg with standard deviation of 5.2mg. This shows that yeasts isolated from tonto are largely fermentative and should be exploited more for ethanol yield with the significant exception of Ca2 and Ca11, with respective yields of 12.8 and 14.1 mg.

Chapter VI. GENERAL DISCUSSION AND CONCLUSIONS

Development of yeast flora

The species diversity of yeast strains associated with fruits, grains and beverages was rather similar in both surface flora and in beverages. The species diversity, in general, was limited. This can be accounted for by several factors. The number of yeasts on surface of fruits and grains were expressed in terms of unit area. These figures were then converted into percentages for purposes of comparison. There is no technique available, such as the keratotomy (Beech and Davenport, 1961) to ensure that all the yeast cells of a known area of surface are removed. All other methods remove but a portion, such as washing and swabbing (Devenport et al, ibid). Swabbing brings in the extra problems of removing yeast cells from the swab. The investigator used washing techniques.

The primary isolation medium should contain the same sugars as are in the substrate (Kaminski, 1958). Yeasts vary in their nutritional and osmotic requirements. ME agar, GYP agar and GYP banana extract agar were used at pH 3.7 to 4.0. To allow Schizosaccharomyces pombe to grow, media for yeasts isolated from beverages were maintained at pH 5-5.7. Differential inhibitors should be used to inhibit strong fermenting yeasts, allowing the isolation of Brettanomyces spp. The worker did not use any inhibitors, except enrichment technique mentioned above. Probably this is responsible for the absence of the genus Brettanomyces in the isolates. A high concentration of vitamins, particularly biotin and thiamine, are necessary to encourage rapid growth of this group of yeasts (van der Walt and Var Kirken, 1960). The investigation had limited supply of biotin and thiamine.

A number of special-purpose enrichment techniques have been devised for the recovery of specific genera or species. These special-purpose techniques rely on the use of inhibitors which only permit the development of the desired species. Some species require use of actidione and sorbic acid at pH 4.8, (Walt & van Kerken, 1961). The specific isolation of Cryptococcus laurentinus require use of creatine in the presence of diphenyl as the source of nitrogen. The development of such technique requires a thorough knowledge of the physiological characteristics of the species it is desired to isolate.

thermophilic species will not be easily isolated unless the temperature is at least 40°C.

The above factors contributed to the limited diversity of species as it was not possible to optimise the conditions owing to scarcity of resources.

There were more colony-forming units of yeasts on banana than on other fruits or grains or ~~the other~~. There is no immediate explanation on account for this. Probably as banana plants (trees) are more closely associated with brewing environment it would be most likely that yeast from the brewing environment provide inocula to the bananas. Large swarms of flies, including the ubiquitous

Sophila, bees, green and other beetles and butterflies were always observed sitting or feeding on ripe banana fruits, which emit aromatic scent and ooze out sweetish exudates, and are often cracked. The same types of insects are observed during brewing of Tonto (Central and Western regions Uganda). Fermentative yeasts constituted at least 50% of the yeast flora isolated from the banana ripe fruit. This may be explained by the fact that fermentative yeasts are quick-growers and tend to be isolated early. The pattern is not so clear from other sources. As Warcup points out, dilution plates always record only a part of the species present on surfaces, or from other extracts of solids.

Fast Strain Physiology

Fast grown axenically (i.e. cultures of a single species of yeasts) on single sterilized medium are unlikely to behave in the same way as they would in a complex environment such as surfaces of fruits, in Tonto, kwete or ajon, where other organisms, such as bacteria and fungi, are present which compete for available nutrients and liberate compounds such as toxins and antibiotics.

Sterilization of artificial media may further complicate interpretation of the results by chemically altering the medium. In addition to these problems of environment and nutrition on yeast behaviour, and hence on yeast taxonomy is a basic difficulty of measurement of growth

absolute measure of growth is dry weight increase, but it may be difficult or, where autolysis occurs rapidly, impossible to separate organisms from the medium in which they are growing. To avoid these problems some other parameter may be measured, e.g. volume growth, sugar growth or increase in optical density of suspensions. However, these parameters have the disadvantage that the value measured may be due to sugar uptake, or vacuolation of pigment production rather than true growth.

In the isolation of yeasts and the study of their cultural morphological and physiological characteristics, relatively crude culture media were used. These media were based on natural products and represented attempts to reproduce the natural environment of the organism (yeast strain) to be studied.

Among the media used in this study were those containing peptones, yeast extract, malt extract, potato extract, banana extract and millet maize extract. All these media are, to varying degrees, crude sources of amino acids, vitamins and organic bases supplemented with sugars (dextrose) and mineral salts. Several liquid media have been used in the study of morphological and physiological characteristics of yeasts.

A wide range of techniques have been used in the study of yeasts on fruits and grains surfaces and those growing and fermenting beverages. Some techniques have been devised to study the yeast population as a whole, others to study a single yeast strain or group of yeasts with a common property, such as fermentative ability or lack of it.

These studies were based on a number of assumptions and experience. The studies depended on making a suspension of surface extract or a dilution of a beverage and incubating a portion of it in an agar medium followed by plate-counts of yeasts obtained from both surface extract suspensions or dilution of fermenting beverages. Any colony on a plate was assumed to have arisen from a single yeast cell. Yeasts are not sporulating fungi (cf. conidia producers such as Aspergillus or Penicillium or Monilia etc...)

difficulty was that certain yeasts, e.g. Brettanomyces are yeasts are slow growers. These were never obtained in dilution studies. Following the realization of the imperfections of artificial media, natural media have been selected and used for isolation of yeasts. These were meant to enlarge the range of yeast strains isolated.

surface extract dilution plate method: This method, the most widely used in soil mycology, has been adopted from the method originally developed for lower "numbers" of surface yeast count. There was no need for lowering dilutions for isolation from beverages. Here counts were as high as bacterial counts from soil. There was modification to adjust the acid reaction to pH 3.7 - 4.0, for suppression of bacteria (Warcup, 1951). The method consisted of shaking a known surface area with sterile water, then obtaining a progressive series of dilutions. From one or more of the dilutions 1 ml samples were placed in Petri dishes and dispersed with melted but cooled agar by shaking and rotating the plate before agar solidified.

These techniques were developed to minimize competition and antagonism among yeast colonies. Even with low numbers, Warcup has found evidence of suppression. Colonies developed always gave significantly higher counts than plates where fungi were allowed to develop normally. Interference was most marked if spreading colonies were present.

To reduce the growth of bacteria and Actinomycetes on dilution plates the medium was adjusted with H_2SO_4 to approximately pH 4. Acid, however, is known to depress or prevent the growth of some yeasts. The use of acid was avoided when plating yeast-rich sources such as fermenting beverages such as TONTO, which usually had pH 5.4.

It can be asserted that the fresh beverages which contain rich sources of nutrients, such as sugars, vitamins and mineral salts gave the highest frequencies of cell forming units. However, as far as biodiversity was concerned, banana fruits gave the highest yeast biodiversity. As the beverages "aged", the reproduction capacity of the yeast strains decreased. The gradual diminution in the colony forming units in time during fermentation it may be necessary to assume that the majority of the yeast strains were being killed by accumulation of ethanol and phenolic compounds and from starvation as the nutrients were exhausted with time, in a batch culture.

strategy of mutant selection is of great importance for applied industrial mycology. It points the way to deliberate selection of high performance mutants for the production of any substances that can be achieved by yeast biological processes.

Before, to this end, 12 yeast strains have been selected for study of special interest in their biological processes, particularly in fermentative abilities and biomass production.

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APPENDICES

NEUBAUER HAEMOCYTOMETER TECHNIQUE

Haemocytometer consists of a special slide with a ruled area in centre, a coverslip and a graduated pipette with a rubber tubing. At low power, X 100 magnification, the central part is ruled. The middle of the ruled area consists of a grid 1mm^2 in area. This is usually called TYPE-A SQUARE, (See figures A-C).

A square is subdivided by lines into 25 TYPE-B squares, each of which has an area of $1/25\text{mm}^2$. Each type-B square is further subdivided by single lines into 16 TYPE-C squares which have an area of 0.0025mm^2 .

The surface of the slide between the two deep grooves is 0.1mm lower than the rest of the slide on either side of the grooves. So when the coverslip is put on, its lower surface clears the ruled surface of the slide by 0.1mm . The volume subtended by the type-A square is therefore 0.1mm^3 . The volume subtended by a type-B square is 0.004mm^3 , and the volume subtended by the type-C square is 0.00025mm^3 .

The pipette has a mark 101 above the bulb.

Procedure for counting yeast cells.

Obtain 1 ml of yeast cells from the batch culture using the rubber tubing attached to the sterile pipette by sucking up the liquid culture up to the "1" mark. Quickly dry the tip of the pipette with sterile filter paper until the liquid culture drops back to the 1 mark.

Suck up distilled water until the contents of the pipette reach the 101 mark. If the 101 mark is passed, the pipette must be emptied and the procedure started again. Care must be taken not to contaminate the culture during the process.

Remove the rubber tubing from the pipette. Close the two ends of the pipette for at least a minute. With the culture thoroughly mixed, blow six drops so as to expel excess water. The yeast culture is now diluted to 1 in 100.

the coverslip in the centre of the slide. Put one drop of yeast culture from the pipette onto the slide alongside the pipette in the area between the two deep grooves. The yeast culture will be drawn under the coverslip by capillary action. Wait five minutes to allow the yeast cells to settle. If the yeast culture flows into the grooves, clean the slide and put on another drop.

Place the slide under the microscope and adjust the illumination so that the grid and the yeast cells can be clearly seen. If the yeast cells are unevenly distributed clean the slide and start again.

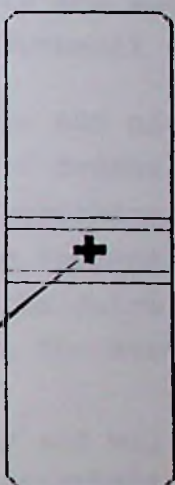
Count the yeast cells in 100 type-C squares are counted and recorded. It should be noted that in each square all the cells which lie entirely within the square, plus those which are touching or overlapping the top and left-hand sides are the only ones that are counted. Do not include cells touching or overlapping the bottom and right-hand sides even if they are within the square.

Calculate the mean number of corpuscles in a type-B square. Bearing in mind the volume represented by type-C square, and the dilution factor, the number of yeast cells per ml in the culture is then calculated.

Example: Supposing the number of yeast cells in type-C square is 10; dilution factor is 101. The volume of type-A square is 0.1mm^3 . The volume of type-C square is 0.00025mm^3 . Since there are 400 type-C squares, then they would contain 10×400 yeast cells.

Their volume is only 0.1mm^3 . So in 1mm^3 there would be $10 \times 10 \times 101$ cells. Now, these yeast cells would be in a volume diluted by a factor of 101. So the actual number of yeast cells in 1mm^3 is $10 \times 400 \times 101 = 4,040,000$ or 4.04×10^6 cells ml^{-1} .

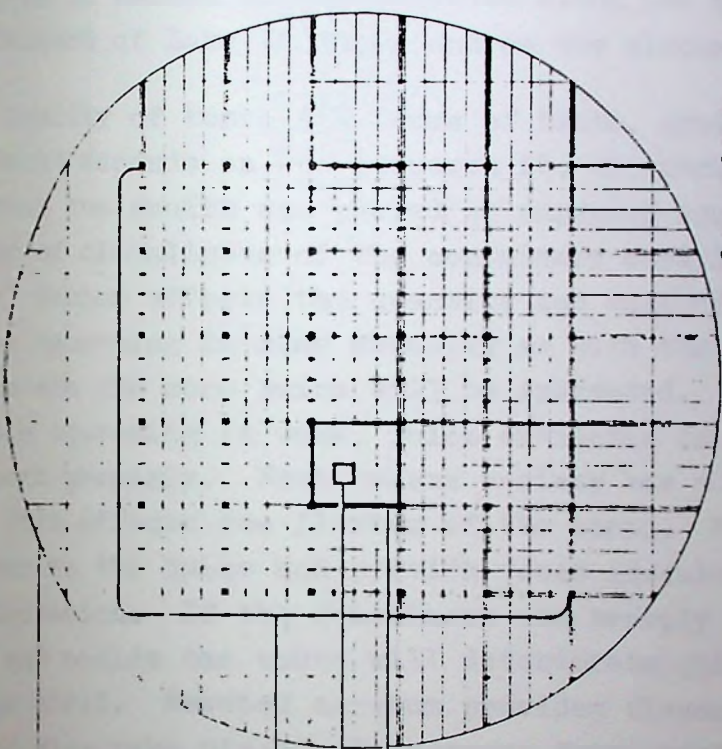
A Surface of slide



B Section of slide showing coverslip in position



C Central part of grid



type-B square
type-C square
type-A square (1 mm side)

edge of field of view (low power)

DESCRIPTION OF BEVERAGES

beverages also called "mwenge bigere" is a major source of alcohol for Uganda waragi distilleries.

Tonto is a designation for sweetish, fermented juice, brown in colour, and a lot of debris of sediments. The juice is extracted from a variety of banana cultivars grown along the shores and in the hinterland of Lake Victoria and on the slopes of Rwenzori and Elgon.

The quality of tonto (in terms of taste, appearance and alcoholic content) depends on who prepares it, the variety of banana used, whether the fruits are peeled or unpeeled during squeezing and the degree of cleanliness of the containers used during fermentation. The "tonto" factor affects the quantity and quality of the juice extracted. When squeezing is done manually or with the feet, the stronger these hands are the more juice will be extracted.

If the squeezing is weak, juice extracted is slimy and will not ferment properly. Each banana variety has a characteristic flavour and this affects the flavour of the tonto. Peelings add flavour and nourish to the juice and provide yeast inoculum, hence, speed of fermentation. If the containers are heavily contaminated with bacteria and moulds the tonto will deteriorate quickly and will have a foul smell. Roasted sorghum provides flavour. Saccharification should also take place and increase fermentable carbohydrate. No effort is made to prevent contamination. The containers used, such as dugouts (canoes), gourds, pots, jerry cans etc.... are not sterile. Preservatives are added. The fermentation process begins almost immediately. The containers are well insulated to conserve heat. Grass trash and banana leaves are used to cover the containers. The juice is left to ferment for 24 hours. The alcohol generated during the ensuing two or three days reached 7 - 8% measured by means of an alcoholimeter. Alcohol is assimilated by bacteria and yeasts such as *Brettanomyces*, etc.... to acetic acid, lactic acid and esters. Filtering is poor so that tonto never clears; is full of residues of grass trash, and sorghum. Tonto is consumed in local bars or distilled locally to produce "crude" gin called waragi or anguli.

DESCRIPTION OF BEVERAGES

10
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investigator intends, in future, to conduct experiments by using sterile conditions of brewing and incubation, controlled sugar content of banana extract, with Hansenula, Saccharomyces and Rhizosaccharomyces both singly and in combination. The brew under these circumstances will be compared with the locally brewed tonto.

MURAMBA

This beverage originated from Kigezi (MRUZJ). The sorghum used is steeped in water for two or three days. It is then drained and placed in layers between banana leaves and allowed to germinate by periodic misting for 4-5 days. Germination continues until the bud is about 5 cm long and the material is then placed under the sun or a clean cloth to dry. It is ground using a mill-stone or a mortar and pestle. The flour is mashed in hot water (50-80°C). The sweetish mash is put in a pot or pipa (metal barrel). A starter mixture of old muramba, mugs of 500 ml each, is always available and is added to each of 20 litres of the mash (porridge). Portions of malted millet flour to sorghum are added. The porridge is left to ferment for 3 - 5 days.

AMARWA: (originally from Eastern Uganda).

In Masaka, Rakai, Mbarara, Ibanda and Rukungiri, amarwa brewing was typically associated with townships of Police Posts. Similar procedure is followed as for muramba, except what proportions of malted sorghum and millet to cassava or maize flour are added but 1:4 is satisfactory. Approximately 25 litres of water are added for about every tin (10 kg) of grain. More millet malt is added to room temperature to make a paste. The mash is put into a pit, lined with banana leaves and well covered with the leaves. After 5-6 days the mash is brown colour. The roasted paste is mixed with a lot of water in a barrel (about 200 litres) to which 5 mugs (2.5 litres) of millet flour are added. Hydrolysis proceeds for 2 hours and fermentation for 2 - 6 days. Amarwa is ready for consumption while it is still actively fermenting. Ethanol is generally between 2 - 4%. The brew contains between 0.3 to 0.6% of acetic acid and variable quantities of esters. Yeast present in the malt flour added afterwards together with species of Mucor and Rhizopus are responsible for hydrolysis and fermentation.

WETE: (Teso, Acholi).

This beverage is precisely the same as Amarwa.

4. DETERMINATION OF RESIDUAL SUGARS

Introduction: During fermentation raffinose (trisaccharide) may be hydrolysed by B-h-fructose as well as by a-galactosidase so that either 1/3, 2/3 or the complete molecule is fermented. Normally during the fermentation of raffinose for 1/3 of the molecule, the fructose moiety (fraction) is fermented with the formation of residual melibiose (a disaccharide) by yeasts, e.g. Saccharomyces cerevisiae which lack a-galactosidase but which possess B-h-fructosidase. Raffinose may, however, also be fermented for 1/3 with the formation of residual sucrose. In this case, which is seldom encountered only the galactose moiety is fermented. This occurs in yeasts, e.g. Saccharomyces aceaeus and Sac. cleaginosus, which possess a-galactosidase but lack either B-h-fructosidase or a-glucosidase active against sucrose.

The fermentation of 2/3 of the raffinose molecule is effected by yeasts which are capable of hydrolysing the raffinose molecule completely, but which lack the ability to utilize galactose, e.g. Sacc.inusitatus and the Sacc. eupagycus. The fermentation of 2/3 of the raffinose molecule is, therefore, associated with the ability to ferment sucrose and also with the ability to ferment half the melibiose molecule.

Table XXVII Fermentation of raffinose

Fermentation of				Assimilation of				Presumed degree of fermentation of the raffinose
Ra	Me	Su	Ga	Ra	Me	Su	Ga	
+	+	+	+	+	+	+	+	Complete
+	+S	+	-	+	+S	+	-	2/3 molecule(residual galactose)
+	-	+	+	+	-	+	+	1/3 molecule(res.melibiose)
+	-	+	-	+	-	+	-	1/3 molecule(res.melibiose)
+	+	-	+	+	+	-	+	1/3 mol.(resid. sucrose)

Key: Ra = raffinose; Me= melibiose, Su=sucrose, Ga-galactose,
S = slow

quently the partial fermentation of raffinose molecule is weak and incomplete so that residual raffinose is present when fermentation is complete. Residual sugars are then best identified by chromatography. An indication of the degree to which the raffinose molecule is fermented by a yeast strain may, therefore, be obtained by inspection of its ability to utilize raffinose, galactose, sucrose and melibiose, Table...

Residual carbohydrate in the case of the incomplete fermentation of raffinose must be determined whether 1/3, 2/3 or the complete molecule is fermented, i.e., whether it is fermented with the formation of residual melibiose, sucrose or galactose, an indication of which may be obtained from Table..... The residual sugars may be identified by chromatography (cf. Minarik et al, 1954), Kockova - Kratochwilova et al, 1966, Donkersloot, 1966 or by microbiological and chemical methods or combination of these methods.

METHODS OF DETERMINING RESIDUAL SUGARS

Microbiological determination of residual sugars

1. Method for the detection of residual melibiose: Skinner C.E. & R. Bonthilet, J. Bacteriol 53:37, 1947.

The raffinose fermentation tube of the yeast under test is incubated 20 days or until the fermentation is complete as indicated by a reduction in volume of gas in the insert tube. An 0.5 ml aliquot of the liquid is withdrawn aseptically from the tube and mixed with 5 ml of Benedict's qualitative reagent. The mixture is boiled for 3 minutes and observed for the presence of reducing sugars. The fermentation tube is then autoclaved for 15 minutes at 10 lbs overpressure. After cooling the tube is re-inoculated with a suspension of Sacc.uvarum which ferments galactose, sucrose, melibiose and raffinose. If carbon dioxide is evolved after re-inoculation and incubation, the Benedict's test is positive for reducing sugar, and the yeast strain under test ferments galactose, then the residual sugar in the fermentation tube may be taken to be melibiose, and the strain ferments 1/3 of the raffinose molecule.

If no reduction by the sugar is detected and no carbon dioxide is evolved after re-inoculation the yeast under test will ferment both raffinose and melibiose completely.

Method for the detection of residual sucrose (van Unde & Assis
Lpex, 1965a).

Procedure described above is followed except that after autoclaving
the is re-inoculated with a suspension of Candida tropicalis which
ferments galactose and sucrose vigorously, but has no action on melo-
bor raffinose. If carbon dioxide is evolved after re-inoculation,
Benedict test is negative for reducing sugars and the yeast under
test utilizes melibiose, then it may be taken that the residual sugar
in the fermentation tube is sucrose and the yeast under test ferments
the raffinose molecule.

Method for the detection of residual galactose (van der Walt,
1965a). The same procedure is followed as in the determination
of residual melibiose, except that after autoclaving the tube
is re-inoculated with a suspension of Sacc. unisporus or Sacc.
diarensis which ferments galactose vigorously but have no action
on either sucrose, melibiose or raffinose. If carbon dioxide
is evolved after re-inoculation the Benedict test is positive
for reducing sugar, and the yeast under test does not utilize
galactose then it may be taken that the residual sugar in the
fermentation tube is galactose and that the yeast under test
ferments 2/3 of the raffinose molecule. In this case melibiose
will be fermented slowly and usually weakly as well.

Quantitative determination of the fermentation of raffinose.

Degree to which raffinose is fermented by a yeast may also be
determined by measuring quantitatively the volume of carbon dioxide
evolved from 4% raffinose solution over sterile mercury in the van
der Kluyver fermentometer (Kluyver, 1914). The determination
takes about 10 days. The volume of carbon dioxide evolved is checked
to see whether it corresponds with either 1/3, 2/3 or complete fer-
mentation of the trisaccharide. If the results are doubtful the
carbon dioxide evolved by the direct fermentation of 4% melibiose
is measured. This method requires specially constructed
apparatus and considerable amounts of clean, sterile mercury.

Chemical determination of residual sugars.

Reagent, chemical method for the determination of the degree to which yeast degrades the raffinose molecule has been described by (1958). This procedure which requires only one fermentation with 2% raffinose per yeast strain, is based on three simple chemical colour reactions. The presence of residual reducing sugar in the fermented liquid is determined directly with Luff's reagent. Portions of the fermented liquid are then hydrolysed and tested specifically for glucose with glucose-oxidase indicator paper (Coomer, 1956) or fructose with methylindol reagent (Malovan 1943). Each fermentation tube contains 2% raffinose in 1% peptone water. Dilute yeast autolysate is inoculated with the yeast under test and incubated until fermentation is complete and no further carbon dioxide is evolved.

Determination of reducing sugar.

1 ml of the fermented liquid is mixed with 5 ml of Luff's reagent in a test tube and boiled for 1 minute over an open flame. If reducing sugar is present, the clear blue liquid turns green and turbid. After a while a yellow or red deposit of cuprous oxide is formed. If reducing sugars are absent the liquid remains blue.

Determination of glucose.

Portions of the fermented liquid are mixed with 1 ml 10% hydrochloric acid in a test tube and heated for 4 minutes in a boiling water bath to effect the hydrolysis of residual sugar. Then 1 drop of methyl orange indicator is added and the solution is neutralized by adding a saturated solution of sodium bicarbonate drop by drop. Glucose indicator paper strips are dipped into the cooled neutral and exposed to air. The strips turn blue if glucose is present, but remain orange if this sugar is absent.

Determination of fructose.

Portions of the fermented liquid are mixed with 0.2 ml methylindol reagent and 3 ml concentrated hydrochloric acid. If fructose is present the light orange solution turns deep red after a few minutes.

The degree to which raffinose is fermented is then determined from the table below.

Table XXVIII Determination of the degree to which raffinose is fermented.

Residual sugar	Glucose	Fructose	Fermentation of raffinose
+	+	-	1/3 (resid. melibiose)
-	+	-	1/3 (resid. sucrose)
+	-	-	2/3 (resid. galactose)
-	-	-	Complete

The chemical tests devised by Bronn for the identification of the residual sugar may of course be used in combination with the microbiological tests described under (a).

KEY TO CODES: A TAXONOMIC STUDY OF YEASTS

ITY = V_{ij} (V_i =District
 V_j village)

SAMPLES = $S_y Y_z$ (Y_z =yeast
strain)

CODE

MR_{ij}/MR_{2j} Sorghum = SO $MR_{ij}/MR_{2j} B_y Y_4$
Banana = B_y Tonto = TO Millet = MI
ie, $S_y = B_y$
Maize = MAI
Pineapple,
 $S_y = po$
Mango: $S_y = M$
Orange: $S_y = O$
ETC.

MRU_{ij}/MRU_{2j} Bushera = BU
Muramba = MU -do- $MRU_{ij}/MRU_{2j} BUY_2$

J^T_{1j}/J^T_{2j} Amarwa = MA -do-

MS_{1j}/MS_{2j} Ajon = MA
Kwete = -do-
Kwete

MEDIA AND REAGENTS

Isolation and maintenance of yeast cultures

Yeast extract-malt extract broth referred to as YM broth (Wickerham, 1951).

Dissolve 3g yeast extract, 3g malt extract, 5g peptone and 10g glucose in 1 litre water. The pH of the medium ranges between 5 and 6 depending on the batch of ingredients. For enrichment purposes the pH is adjusted to 3.7 with dilute hydrochloric acid. After dispensing, the medium is sterilized at 15 pounds overpressure for 15 minutes.

Yeast malt extract agar, referred to as YM agar (Wickerham, 1951). This medium is prepared by adding 2% agar to yeast extract-malt extract broth before sterilization. The pH of the medium varies from 5-6. The medium is sterilized at 15 pounds overpressure for 15 minutes.

Malt extract (Lodder & Kreger van Rij, 1952).

To make malt extract, 1kg of malt mixed with 2.6 litres tap water is heated in a water bath at 45°C for 3 hours and is stirred continuously. The temperature is then raised to 63°C and maintained for 1 hour, after which the mixture is pressed through a hair sieve or cheese cloth. The filtrate, after being autoclaved for 15 minutes at 15 pounds overpressure is filtered through a paper and diluted to a density of 15° Balling, the pH being adjusted to 5.4 if this is necessary. When this malt extract is used for enrichment purposes the pH is adjusted to 3.7 after which it is dispersed and sterilized for 15 minutes at 10 pounds overpressure.

Sometimes it is possible to obtain unhopped malt extract from breweries. The wort is adjusted to pH 5.4 and then diluted to 15° Balling. When malt extract is used for enrichment purposes the pH is adjusted to 3.7. After dispensing, the malt extract is sterilized for 15 minutes at 10 bls overpressure.

If brewery wort is not available powdered malt extract may be used. Dissolve 10% (w/v) powdered malt extract in distilled water by heating in a boiling water bath. After cooling the extract is adjusted to pH 5.4.

Malt agar

Malt extract is diluted to 10° Balling to which 2% agar is added. After dispensing, the medium is sterilized at 10 lbs overpressure for 15 minutes.

Cultural characteristics: Same for isolation and maintenance of yeast culture (see A and E.1X).

Morphological Characteristics

1. Characteristics of Vegetative Cells

1. Malt extract (See A)

2. 2% (w/v) glucose yeast-peptone water. Dissolve 20g glucose, 10g Bacto-peptone and 5g Bacto-yeast extract in 1 litre of distilled water. The pH is not adjusted. After dispensing, the medium is autoclaved for 15 minutes at 15 lbs overpressure.

3. Malt agar (see A).

4. 2% (w/v) glucose-yeast extract - peptone agar. This medium is prepared by adding 2% (w/v) agar to 2% (w/v) glucose-yeast extract peptone water. After dispensing, the medium is autoclaved for 15 minutes at 15 lbs overpressure.

5. Morphology agar

The composition of this medium is given in Table

To rehydrate the medium, suspend 35g of Bacto-yeast morphology agar in one litre of cold distilled water. Heat to dissolve the ingredients completely. Disperse in tubes or flasks and sterilize by autoclaving for 15 minutes at 15 lbs overpressure. The final reaction of the medium is about pH 5.6.

Formation of pseudomycelium and true mycelium.

1. Corn meal agar (Bernhardt, 1946): 12.5g yellow maize meal is stirred in 300 ml water and heated in a water bath at 60°C for 1 hour and then filtered through paper. The volume of the filtrate is made up to 300 ml and 3.8g agar is added. The mixture is autoclaved for 15 minutes at 15 lbs overpressure. The hot liquid is refiltered through absorbent cotton wool, dispensed and sterilized for 15 minutes at 15 lbs overpressure.

Other modified methods for the preparation of maize meal agar have been described by Benham (1931), Abello (1945), Skinner (1947), Kligman (1950),

Gordon et al (1952). Suitable commercial brands of maize meal are also available.

Potato-glucose agar

20g washed, peeled and finely grated potatoes are soaked in 300 ml water for several hours in a cold room or refrigerator. The mass is filtered through cloth and autoclaved for 1 hour at 15 lbs overpressure. For the preparation of the agar, 230 ml of this potato extract is added to 770 ml tap water containing 20g glucose and 20g agar. After the medium is dispensed and sterilized for 15 minutes at 15 lbs overpressure. For the preparation of the agar, 50 ml of this potato extract is added to 770 ml tap water containing 20g glucose and 20g agar. After the medium is dispensed and sterilized for 15 minutes at 15 lbs overpressure.

Morphology agar (Wickerham, 1951). See C.

Formation of Chlamydospores

- . Malt agar (see A)
- . Potato agar (see C)
- . Maize meal agar (see C)

Sexual Characteristics

- . Presporulation medium, medium of Lindgren (1940). This medium contains 10 ml beet leaves extract (100g per 100 ml boiling water), 10 ml beetroot extract (100 g per 100 ml boiling water), 35 ml canned apricot juice, 16.5 ml bottled grape juice, 2g dried yeast, 2.5 ml grape juice, 1 g calcium carbonate and 3 g agar. Water is added to give a final volume of 100 ml. After solution of the agar by steaming for 10 minutes, the medium is dispensed in tubes and autoclaved for 15 minutes, 15 lbs overpressure.

Sporulation media

1. Vegetative wedges: carrot-potato, beet and cucumber wedges may be used. The vegetables are thoroughly cleaned by washing. Long cylinders about 1 cm in diameter are cut from the vegetables with the aid of a cork borer. Wedges are prepared by cutting the cylinders obliquely. The wedges are rinsed with cold water and placed in suitable test tubes containing some water to prevent desiccation. The tubes are sterilized by autoclaving for 15 minutes at 10 lbs overpressure.

vegetable juice agar (Mrak et al, 1942). Equal weights of washed, peeled carrots, beet roots, cucumbers and potatoes are minced or ground finely and mixed with a quantity of water equal to the weight of vegetables used. The mixture is autoclaved and expressed through a cloth. The extract has pH of circa 5.7 and is about 4° Brix. To the extract is added 2% dried yeast and 2% agar. After dissolution of the agar by steaming, the medium is dispensed in tubes and autoclaved for 15 minutes and at 15 lbs overpressure. Wickerham (1946) recommended the following vegetable juice agar: 14g of agar is melted in 340 ml distilled water; in another container is added 350 ml of V8 juice (this is canned vegetable juice containing a blend of tomatoes, carrot, celery, beets, parsley, lettuce and onion), 5g of moist compressed yeast dispersed in 10 ml distilled water. The suspension is stirred. The pH is adjusted to 6.8. The suspension is then heated for 10 minutes in flowing steam. The pH is again adjusted until a cooked sample has pH of 6.8. The medium is then poured into the hot agar solution, stirred and dispensed into tubes. Sterilization is at 15 lbs overpressure for 15 minutes. Slants are prepared as needed, avoiding unnecessary heating when melting the medium.

Wickerham agar (modified).

To distilled water add 0.1 (w/v)% glucose, 1% peptone, 0.5% sodium chloride and 2% agar. Steam until the agar dissolves and dispense in tubes. Autoclave for 15 minutes at 15 lbs pressure and slant. Some workers substitute 0.25% glucose and the addition of 1% meat extract.

Fewell's acetate agar (Fewell, 1952): To distilled water add 0.5% sodium acetate trihydrate and adjust the pH to 6.5 - 7.0. Add 2% agar and steam to dissolve the agar. Dispense in tubes, autoclave for 15 minutes at 15 lbs overpressure and slant.

Wickerham's acetate agar (Wickerham, 1954): To distilled water add 0.25% (w/v) Bacto-tryptose, 0.062% glucose, 0.062% sodium chloride, 0.5% sodium acetate and 2% agar. Dissolve the constituents by steaming and dispense in tubes.

Sterilize by autoclaving for 15 minutes at 15 lbs overpressure and slant. Initial pH after sterilization is 6.9 - 7.1.

McClary's acetate agar (McClary et al 1959): To distilled water add 0.1 (w/v)% glucose, 0.18% potassium chloride, 0.25% yeast extract, 0.25% sodium acetate trihydrate and 1.5% agar. Dissolve the constituents by heating and dispense in tubes.

sterilize by autoclaving for 15 minutes at 15 lbs overpressure and

See also Galzy & Bizeau, 1966; Iverson, 1967.

extract agar or ME agar (Wickerham, 1951). Dissolve 12 g agar
400 ml distilled water by steaming. Then dissolve 20 g powdered
extract in the hot solution. Dispense in tubes or bottles and
sterilize by autoclaving for 15 minutes at 15 lbs overpressure and
at. Slants are kept in the refrigerator until used. Minimal
flaming should be used in melting the sterilized medium.

ato-glucose agar (see C).

ze meal agar (see C).

agar (see A).

st extract - glucose agar: To distilled water add 0.5 (w/v)%

a commercial powdered yeast extract, 5% glucose and 2% agar.

solve by steaming and dispense in tubes. Sterilize by autoclaving
15 minutes at 15 lbs overpressure and slant.

ast infusion (yeast water) agar: To yeast infusion add 1.5% agar.

sterilize by autoclaving for 15 minutes at 15 lbs overpressure.

gypsum blocks (calcium sulphate hemihydrate) with three parts of
water. Cast the paste into cylindrical or truncate conical forms
about 3 - 4 cm high. After setting the blocks are placed in suitable,
sterile glass dishes with covers and maintained at 110 - 120°C for
at least 2 hours. Before use, sterile water is added to the dishes
to give a depth of about 1 cm. Saito (1923) advocates the use of
a solution of a mannitol-dipotassium phosphate mixture (18 ml 0.5-
mannitol + 2 ml 5% K_2HPO_4). A thin layer of well nourished
yeast cells is spread on the top of the block.

gypsum may also be placed in tubes. Gypsum and water are mixed
to a creamy paste which is poured in sterile cotton-plugged tubes
by means of a funnel. The tubes are then slanted and allowed to
harden at 50°C for 24-48 hours.

The plugged tubes are then autoclaved.

Many variations of the gypsum block technique have been tried.

Cartelius and Ditleusen (1953) prescribed the use of cement blocks.

Other variations involve the use of fire brick blocks, clay tubes,
filter paper and even wood. None of these, however, appears more
effective than gypsum.

Markey's ethanol medium (modified). This medium contains 0.5% ethanol, 0.1% dipotassium hydrogen phosphate, 0.025% potassium hydrogen phosphate, 0.025% magnesium sulphate heptahydrate, 0.005% calcium chloride and 2% agar in tap water. Autoclave for 15 minutes at 14 lbs overpressure. Ethanol is added after sterilization.

fertility in ascomycetous yeasts as a taxonomic criterion.

sporulation media in (D).

characteristics of the teliospores and sporidia.

Aqueous agar

Dissolve 20g agar in 1 litre distilled water. Dissolve and autoclave at 10 - 15 lbs overpressure.

Ammonium sulphate agar. Aseptically add 0.15 ml sterile 10% ammonium sulphate solution and 1 drop of the hundred fold concentrated filtered sterilized vitamin solution to 15 ml sterile 2% washed agar in distilled water, cooled to 50°C. Mix the solution shaking and pour plates.

Maize meal agar (See C)

Malt agar (see A)

Rice agar (Tashidjan, 1953): cover 20g of white rice with 1 litre tap water and bring to boil. Allow to boil over a low flame for 45 minutes, keeping the vessel covered. Filter the liquid through gauze.

Discard the rice. After cooling add tap water to the filtrate to make up 1 litre. Add 20g of agar and heat the mixture in a water bath or over a low fire to dissolve the agar. Distribute in tubes or bottles and autoclave the medium for 15 minutes at 15 lbs overpressure.

Biological Characteristics

Utilization of carbon compounds: General media

Yeast autolysate: Mix equal weights of compressed yeast and water in a flask. A thin layer of toluene may be added. Incubate at 50 - 55°C for 3 days stirring occasionally. Boil for a few minutes and allow to cool. The pH is adjusted to 6 by the addition of N KNOH. The supernatant is cleared either by the addition of egg white or by filtration with use of filter aid, e.g., Hyflo Supercel.

clear filtrate represents full strength yeast autolysate.

Yeast infusion or yeast water (Lodder & Kreger-van Rij, 1952).

Mix 200g compressed yeast with 1 litre tap water, add some egg white and autoclave at 15 lbs overpressure for 15 minutes. The mixture is filtered while hot through thick filter paper, twice if necessary.

Fermentation basal medium (Wickerham, 1951).

Dissolve 4.5g powdered yeast extract (a batch should be used which will support the growth of markedly vitamin-deficient yeast e.g., *Kloeckera apiculata*) and 7.5g peptone in 1 litre distilled water. Add sufficient bromothymol blue to give a sufficiently dense green colour. Two ml aliquots are placed in 150 x 12 mm tubes carrying insert tubes. The tubes are sterilized by autoclaving for 15 minutes at 15 lbs overpressure. On cooling the 1 ml concentrated, filter-sterilized sugar solutions are added aseptically.

Stock vitamin solution (van der Walt & van Kerken, 1961).

Dissolve 0.2 mg biotin, 40mg calcium pantothenate, 0.2 mg folic acid, 200mg inositol, 40 mg niacin, 20mg p-aminobenzoic acid, 40mg pyridoxine hydrochloride, 20mg riboflavin and 100mg thiamine in 1 litre distilled water and sterilize by filtration. The sterilized solution is dispensed in 10 ml aliquots and stored at - 10 to -15°C.

Reagents

Benedict's reagent (Browne & Rehman, 1941): Dissolve 173g of sodium citrate and 100g anhydrous sodium carbonate in about 800 ml distilled water and 17.3 g cupric sulphate pentahydrate in 100 ml of distilled water. Mix the solutions and make up to 1 litre.

Luff's solution (Schoorl, 1929): 50g citric acid and 143g anhydrous sodium carbonate are dissolved in 400ml distilled water. Mix with a solution of 25g cupric sulphate pentahydrate in 100ml distilled water make up to 1 litre. The filtered solution may be kept indefinitely.

Use-indicator papers: These are obtainable under the trade
"Glukotest" (Boehringer and Sohne GmbH, Mannheim, West Germany)
"Mistrix" Ames Company Inc., Elkhart, USA; or "Tes - Tape", Eli
Co. Indianapolis USA.

Indol reagent: 0.5% solution of 3-methylindol in 90% ethanol.
Chloric acid, 10%.

Chloric acid, concentrated (D 1.9).

Trated, aqueous solution of sodium bicarbonate.

yl orange indicator: 0.1% aqueous solution.

Utilization of Carbon compounds:

Cultivation medium (Wickerham, 1951): Dissolve 6.7g of Bacto-yeast
Nitrogen base, See Table..... and 1g glucose in tenfold conce-
ntrated solution is filter-sterilized and is stored in the refri-
gerator. One ml aliquots of this basal medium are then pipetted
9 ml amounts of sterile distilled water in cotton-plugged 16mm
tubes.

Stock vitamin solution (van der Walt & van Kerken, 1961), see E4.

Nitrogen basal medium for carbon assimilation tests in liquid medium

The composition of the basal medium is given in Table..... A tenfold

concentrated medium is prepared by dissolving 6.7g of Bacto-

yeast nitrogen base and the appropriate amount of the carbon comp-

ound equivalent to glucose (i.e., containing the same amount of

carbon as 5g glucose in 100ml of distilled water). When raffinose

is the carbon source it is used at twice this concentration. It

may be necessary to warm the distilled water somewhat to effect

complete solution. When organic acids or their acid salts are

used as the carbon source, the pH of the solution is adjusted to

6.5 by the addition of sodium hydroxide pellets. The solution is

then sterilized by passing through a Seitz filter and the sterile

solution is kept in the refrigerator until required. Aliquots of

0.5 ml of the sterile solution are pipetted aseptically into clear,

cotton-plugged 16mm tubes containing 4.5ml distilled water which

have been sterilized by autoclaving for 15 minutes at 15 lbs over-

pressure. After shaking the tubes are ready for use. The following

precautionary measures are taken.

When soluble starch or inulin is used as the carbon source, it is

dissolved in distilled water by gently heating over an open flame

otherwise it may be lost to a considerable extent if Seitz

filters were used for sterilization.

solution contains 0.5% of the carbohydrate are then pipetted 0.5 ml amounts into sterile cotton-plugged 16 mm tubes. The tubes are sterilized by autoclaving for 15 minutes at 10 lbs over-pressure. After cooling each sterilized tube receives 0.5 ml of the basal medium in tenfold concentration. After shaking the tubes are ready for use.

Certain oligosaccharides e.g. sucrose and maltose, may undergo hydrolysis (See NB below) in the basal medium on prolonged storage, some laboratories prefer to filter-sterilize the basal medium and carbohydrate solutions separately. For this purpose, Bacto-yeast nitrogen base is dissolved in 100 ml distilled water and the solution is sterilized by filtration. The solution is distributed aseptically in 0.5 ml amounts in sterile, cotton-plugged 16mm tubes. Sufficient of the carbohydrate to make up 100ml of the finally diluted medium (i.e equivalent to 0.5 g glucose) is dissolved in 90 ml of distilled water and filter-sterilized. The final medium is then prepared by aseptically adding 4.5 ml of sterile carbohydrate medium to each of 0.5 ml aliquots of sterile basal medium.

Hydrolysis of sucrose for instance may be detected by testing for assimilation and growth with any yeast which does not utilize sucrose. Solutions which have undergone hydrolysis should be rejected.

Utilization of Nitrogen Compounds

High grade, preferably analytical grade compounds should be used as nitrogen sources in the nitrogen assimilation tests. When using amine salts it is advisable to ascertain that these are free of traces of ammonium compounds.

Assimilation in liquid medium (Wickerham, 1951): The liquid medium employed for testing the ability of yeasts to utilize the various nitrogen sources is similar in composition to that used in tests on carbon assimilation. Its composition is given in

Table.....

The medium is prepared in a tenfold concentration by dissolving 7.5 g Bacto-yeast carbon base together with the requisite amount of the nitrogen source in 100 ml distilled water.

The nitrogen source is a nitrate, 0.78g potassium nitrate is used when it is a nitrite, 0.26g sodium nitrite is used and where a primary amino acid, 0.64g ethylamine hydrochloride. It is necessary to warm the water somewhat to effect complete solution which is then filter - sterilized by passing it through a Gitz filter; it is stored in the refrigerator until required. Final medium is prepared by transferring aseptically 0.5 ml aliquots of the sterile tenfold concentrated medium into 4.5 ml aliquots of sterile distilled water in 16mm cotton-plugged tubes, which have been autoclaved for 15 minutes at 15 lbs overpressure. The tubes are prepared by adding 0.5ml of a filter-sterilized 1% solution. After the tubes have been shaken to mix the contents and the denser, tenfold concentrated solutions, the media are ready for use.

Reagents

Reagent 1.

Dissolve 8g sulphanilic acid in 1 litre of 5 N acetic acid (1 part concentrated acetic acid to 2.5 parts of water) or in 1 litre of dilute sulphuric acid (1 part concentrated acid to 20 parts of water).

Reagent 2

Dissolve 5g α -naphthylamine in 1 litre 5N acetic acid or in dilute sulphuric acid (see above), or dissolve 6 ml of dimethyl- α -naphthylamine in 1 litre of 5N acetic acid. The latter reagent gives a permanent red colour in the presence of high concentration of nitrite.

Add a few drops of each of these reagents in nitrate cultures. The development of a distinct pink or red colour indicates the presence of nitrite.

Growth on media of high osmotic pressure

Media

Growth on 50% and 60% (w/w) glucose-yeast extract agar. The two media are made up by dissolving 50g and 60g of glucose in 50ml and 40ml respectively of either yeast infusion or 1% yeast autolysate. Note the volume. Add 3% (w/v) agar and dissolve by steaming or in a waterbath. After dissolving the agar, the medium is dispensed in 5 - 6 ml aliquots in 16mm cotton-plugged tubes.

The tubes are autoclaved for 15 minutes at 10 lbs overpressure and then slanted.

Growth at moderate osmotic pressure. Dissolve 5g glucose and 10g sodium chloride in 100 ml of distilled water. The solution is dispensed in 4.5 ml aliquots in 16mm cotton-plugged tubes and sterilized by autoclaving, for 15 minutes at 15 lbs overpressure. To each tube is added 0.5ml of a filter-sterilized solution of 6.7g Bacto-yeast nitrogen base dissolved in 100ml of distilled water. The solutions are mixed by shaking and are ready for use.

Sodium chloride tolerance test.

Basal medium: 2% (w/v) glucose, 1% (w/v) peptone and 0.5% (w/v) powdered yeast extract dissolved in water. The final medium is dispensed in tubes and sterilized by autoclaving.

The test is performed in sets of cotton-plugged tubes containing 5ml of the basal medium to which sodium chloride is added in 1% (w/v) increments to give a range of concentrations varying from nil to saturation.

Growth at elevated temperatures

Media

Growth at 37°C

1. Liquid medium. A tube of the usual glucose assimilation medium.
2. On solid medium (Kreger van Rij, 1964).
YM agar; malt agar.

Maximum temperature for growth (van Uden & Farinha, 1958).

Acid production

Medium

To 1 litre yeast infusion or dilute (1:10) yeast autolysate or 0.5% powdered yeast extract in distilled water, add 50g glucose, and 5g chalk (calcium carbonate precipitated light) and 20g agar. Sterilize by autoclaving for 15 minutes at 15 lbs overpressure. Cool to 50°C, swirl gently to suspend the chalk evenly throughout the medium. Distribute aseptically in tubes and slant on a cold surface to avoid settling of the chalk.

Production of extracellular, amyloid compounds.

1. Medium A : (Lodder & Kreger van Rij, 1952).

Dissolve 0.2% ammonium sulphate, 0.2% potassium dihydrogen phosphate, 0.1% magnesium sulphate heptahydrate and 2% glucose in distilled water.

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Adjust the pH of the solution to 4.5. Prepare an equal volume of 4% agar in distilled water. The two separate portions of the media are autoclaved for 15 minutes at 10 lbs overpressure. Add the two portions together aseptically while the agar is still liquidified. Gently mix the solutions to avoid frothing. Prepare plates in Petri dishes containing 1 drop of yeast autolysate and 1 drop of a hundred fold concentrated vitamin solution.

If slants are required the growth factor supplement is added to the liquefied agar which is then dispensed aseptically in 5 - 6 ml aliquots in sterile cotton-plugged tubes.

I. Medium B

Dissolve 10g glucose and 6.7g Bacto-yeast carbon base in 100ml distilled water and filter-sterilize. Add aseptically $\frac{1}{2}$ volume of the tenfold concentrated medium to $4\frac{1}{2}$ volumes of sterile liquidified 2 $\frac{1}{2}$ % agar. The solutions are mixed and plates or tubes poured aseptically.

Reagent

Lugol's iodine solution C (Gram's modified).

Dissolve 1g iodine and 2g potassium iodine in 300 ml distilled water.

III Hydrolysis of urea

Christensen's urea agar (Christensen, 1946). Dissolve 1g peptone, 1g glucose, 5g sodium chloride and 2g potassium dihydrogen phosphate and 0.012g phenol red in 1 litre distilled water and adjust the reaction to pH 6.8. Add 20g agar and dissolve after which 4.5 ml aliquots of the medium are dispensed in 16mm cotton-plugged tubes. Autoclave for 15 minutes at 15lbs overpressure. Immediately after autoclaving 0.5ml of a 20%, filter-sterilized urea solution is added. After mixing the tubes are slanted and allowed to set.

Christensen's medium is also marketed in a convenient, sterilized form by the Difco Laboratories (Detroit, Michigan) as Bacto-urea agar base concentrate. To prepare the medium dissolve 1.5g of Bacto-urea agar in 90ml distilled water. Sterilize by autoclaving for 15 minutes at 15 lbs overpressure. Cool to 50-55°C and add the contents of one tube filter-sterilized Bacto-urea agar base concentrate (10ml) under aseptic conditions.

thoroughly and distribute aseptically in sterile cotton-plugged
es.

Pigment formation

Pigmentation is normally observed in growth on the media employed for determining the cultural properties of yeast strains. However, for the production of pulcherrimin by Candida pulcherrima Beijerinck's medium (as modified by van der Walt (1952) gives the best results. Dissolve 4g sucrose, 2g Bacto-peptone, 0.2g potassium dihydrogen phosphate, 0.005g magnesium sulphate heptahydrate, 1 ml yeast autolysate and 2g agar in 99ml tap water. After autoclaving, 1 ml of a sterile 5% solution of ferric ammonium citrate (Merck's ferri ammonium citricum fusc in lammelis) is added aseptically,

X Ester production

Media

1. YM agar
2. 20ml 5% glucose solution in yeast infusion. Dissolve 5(w/v)% glucose in a yeast infusion; dispense in cotton-plugged flasks; autoclave for 15 minutes at 15 lbs overpressure.

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Composition of chemically defined media¹, for taxonomic study of yeasts

Formula Ingredients per liter	Bacto-yeast morphology agar	Bacto-yeast nitrogen base for carbon assimilation tests	Bacto-yeast carbon base for nitrogen assimilation tests	Bacto- vitamin free yeast base for vitamins require- ment test
Nitrogen sources:				
Ammonium sulphate	3.5g	5g	none	5g
Bacto-asparagine	1.5g	none	none	none
Carbon source:				
Bacto-dextrose	10 g	none	10 g	10 g
Amino acids:				
L-Histidine monohydrochloride	10 mg	10 mg	1 mg	10 mg
DL-Methionine	20 mg	20 mg	2 mg	20 mg
DL-Tryptophane	20 mg	20 mg	2 mg	20 mg
Vitamins:				
Biotin	20 µg	20 µg	20 µg	none
Calcium pantothenate	2000 µg	2,000 µg	2,000 µg	none
Folic acid	2 µg	2 µg	2 µg	none
Inositol	10,000 µg	10,000 µg	10,000 µg	none
Niacin	400 µg	400 µg	400 µg	none
p-aminobenzoic acid, Difco	200 µg	200 µg	200 µg	none
Pyridoxine hydrochloride	400 µg	400 µg	400 µg	none
Riboflavin	200 µg	200 µg	200 µg	none
Thiamine hydrochloride	400 µg	400 µg	400 µg	none
Compounds supplying trace elements:				
Boric acid	500 µg	500 µg	500 µg	500 µg
Copper sulphate	40 µg	40 µg	40 µg	40 µg
Potassium iodide	100 µg	100 µg	100 µg	100 µg
Ferric chloride	200 µg	200 µg	200 µg	200 µg
Manganese sulphate	400 µg	400 µg	400 µg	400 µg
Sodium molybdate	200 µg	200 µg	200 µg	200 µg
Zinc sulphate	400 µg	400 µg	400 µg	400 µg
Salts:				
Potassium phosphate monobasic	0.85 g	0.85 g	0.85 g	0.85 g
Potassium phosphate dibasic	0.15 g	0.15 g	0.15 g	0.15 g
Magnesium sulphate	0.5 g	0.5 g	0.5 g	0.5 g
Sodium chloride	0.1 g	0.1 g	0.1 g	0.1 g
Calcium chloride	0.1 g	0.1 g	0.1 g	0.1 g
Bacto-agar	18 g	none	none	none
Amount of final medium from 100g dehydrated medium	2.81	14.91	8.51	5.9
Amount of dehydrated medium per lt. of finished	35.0g	6.7 g	11.7 g	16.7

UGANDA

