RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) IN PALMWINE YEAST TAXONOMY

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A THESIS SUBMITTED

BY

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IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE
MASTER OF PHILOSOPHY DEGREE.

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September, 1994

DECLARATION.

The experimental work described in this thesis was performed by me, at the DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA, LEGON under the supervision of Prof. K. K. Oduro. I declare that this work has not being previously accepted for any degree and is not being submitted in candidature for any other degree.

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DEDICATION.

BLESSED BE THE LORD GOD ALMIGHTY, FOREVER AND EVER.
THINE , O LORD, IS THE GREATNESS, AND THE POWER, AND THE GLORY, AND THE VICTORY, AND THE MAJESTY.
BOTH RICHES AND HONOUR COME OF THEE, AND THOU REIGNEST OVER ALL; AND IN THINE HAND IS POWER AND MIGHT; AND IN THINE IT IS TO MAKE STRONG AND TO GIVE STRENGTH UNTO ALL.
NOW THEREFORE, OUR GOD, WE THANK THEE , AND PRAISE THY GLORIOUS NAME.

To all members of the Brown family of Osu Blogodo especially Mr E.F.A. Brown. To Sue and Bee, Mum and all Nii " Atians". Thank you for your love and care.

ACKNOWLEDGEMENTS.

I am glad to have this opportunity of expressing my deepest gratitude to my supervisor, Dr. K. K. Oduro, who provided guidance and encouragement throughout the period of this work, and for giving me access to his Mac. His invaluable comments have also helped to greatly improve the quality of the work. Special thanks goes to all members of the Oduro family for their warm receptions.

A word of appreciation goes to all the lecturers of the Biochemistry Department for their continued interest and encouragement. My sincere thanks also goes to all the technical staff of the department and to Mike of the Nutrition and Food Science Department, all who in diverse ways helped make this work a success. I would also like to thank my coursemates, Lambie and Batho, whose co-operation I enjoyed. Thanks also to my "Biochem" mates, Clifford, Achel, Kamassah, Crabbe, William and also my roommates, Rodger, Francis and Ebo, whose support kept me going.

Finally, but by no means the least, I thank my very good friends Judith, Vanessa, Palmel, Ethel, Harry, Micky, George, Emily, Mike, Laura, Belinda "Y", Mr's Asare-Yeboah, Joyce Adom, all members of the CCF Counselling Department, Prayer Force and Music Department and all pastors of Grace Outreach Church for their encouragement, moral support and prayers in times of frustration.

GOD BLESS YOU ALL.

ABSTRACT.

Reports on the number of isolates and yeast species obtained from palmwine samples have differed very widely. One factor that probably accounts for this is the method of phenotypic discontinuity which has exclusively been employed in palmwine yeast speciation. The phenotypic features used mainly for this kind of speciation have been shown to be unstable. Consequently it was decided to employ the more consistent and reliable method of Restriction Fragment Length Polymorphism (RFLP) to ascertain the veracity or otherwise of the literature reports on palmwine yeast isolates.

Out of the ten palmwine yeast samples carefully selected from different localities in Southern Ghana, nine showed only one yeast isolate while the tenth sample (from Legon village) showed two isolates. All the yeast isolates were identified as *Saccharomyces cerevisiae*.

The genomic DNA restriction fragment patterns of all the palmwine yeast isolates were examined following the isolation and purification of their DNAs, restriction enzyme digestion of the isolated DNAs, and 0.7% agarose gel electrophoresis at 100 V for 1.3 hours. While the nine isolates PW/B/1, PW/B/2 through to PW/B/9, showed identical patterns, irrespective of any of the seven restriction endonuclease enzymes, *Apa* I, *Bam*H I, *Eco*R I, *Hind* III, *Kpn* I, *Pst* I and *Sma* I used, the patterns of the two isolates from Legon differed slightly from the other nine, but were identical to each other. From these results, it was concluded that isolates PW/B/1, PW/B/2 through to PW/B/9, are the same species, while the other two, PW/B/10a and PW/B/10b are either subspecies or strains of these nine.

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ABBREVIATIONS

A (A_{260})	absorbance (absorbance at 260 nm)
A	adenine
bp	base pair
C	cytosine
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Fig.	Figure
g	gravity (multiples of, as in centrifugal field)
G	guanine
Kb	kilobase
PCR	Polymerase Chain Reaction
ppm	parts per million
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
S.	*Saccharomyces*
sp.	species
T	thymine
U	uracil
UV	ultraviolet
v/v	volume/volume (concentration)
W. H. O.	World Health Organisation
w/v	weight/volume (concentration)

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) IN PALMWINE YEAST TAXONOMY.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The standard description of a species gives the characteristics by which an organism is recognized. These characteristics are, in general, similar for all species. They are morphological, physiological, biochemical and sometimes ecological. The taxonomic value of the descriptive characters depends (a) on their ability to differentiate and (b) on their being constant (Kreger-van Rij, 1987). Most of the characters for microbiological speciation are defined as the result of a particular test, eg. fermentation and assimilation. The weaknesses inherent in such speciation based exclusively on phenotypic differentiation have become apparent. Yeast taxonomists began to realize that phenotypic features, notably fermentation and assimilatory properties, could be unstable (Scheda and Yarrow, 1966, 1968). This holds, not only for physiological and biochemical properties, but also for morphological features which may change with the medium of growth. Research into the chemical composition, the ultrastructure and nuclear DNA base composition of yeasts has added characters to the standard descriptions which are considered to be of fundamental importance to classifying yeasts (Kreger-van Rij, 1987).

The study of genetic compatibility of organisms as well as the information macromolecules (nucleic acids and proteins) which are ultimately responsible for the expression of the phenotypic traits, has long been considered a more meaningful approach to speciation (Marmur *et al.*, 1963). Proteins and nucleic acids are the only molecules that carry enough information in their sequences to measure the totality of diversity of any group (Grimont and Grimont, 1991).

Modern molecular biology techniques, which are very simple to master, have made nucleic acids the preferred macromolecules for speciation; since nucleic acids can be sequenced relatively easily and, even when undetermined, their sequences can be quickly compared by molecular hybridisation. In addition, there are short sequences of double-stranded DNA that are recognized by restriction enzymes as cleavage sites. The number and position of these endonuclease-specific restriction sites on a DNA molecule determine the number and size of the fragments generated by cleavage. The development of simple analytical methods for the separation of DNA fragments by agarose gel electrophoresis allows the comparison of restriction patterns. By following the pattern of production of the restriction fragments, a restriction map can be produced with the located sites on the DNA that are attacked by the restriction enzyme.

Restriction maps are very useful in comparing portions in genomes, since exact correspondence suggests that two fragments of DNA are identical or have arisen by recent duplication of a portion of the genome. Loss or gain of a restriction site may result from a point mutation, or deletion or insertion of bases. Price *et al.,* (1978) maintain that "if two organisms are related, they must retain in their genomes, base sequences that are descendant from a common ancestral base sequence; closely related organisms will have retained a greater portion of base sequences in common, than organisms that have highly diverged."

Although the study of yeast variation at the level of the genomic DNA reveals new types of variability that are not detected by phenotype, karyotype or enzyme analysis, it is only comparatively recently that this approach to yeast speciation has been developed. The study of yeast variation at the level of the genomic DNA has its origin in modern developments in bacterial systematics resulting from comparison studies of prokaryotic genomes (Marmur *et al.,* 1963; Mandel, 1969) and on an assessment of relatedness by means of nucleic acid analyses. It was only subsequent to this development in bacterial systematics, and chiefly as a result of many initial studies (Belozersky and Spirin, 1960; Storck, 1966; Storck *et al.,* 1969; Bickness and Douglas, 1970) that genome comparison studies were found to have taxonomic significance for yeasts.

The yeast, *Saccharomyces cerevisiae* is recognized as an ideal eukaryotic microorganism for genetic studies (Sherman, 1991). Some of the properties that make yeasts particularly suitable for genetic studies include rapid growth, a well defined genetic system, and most importantly, a highly versatile DNA transformation system. Being nonpathogenic, most *Saccharomyces* species can be handled with minimum precautions.

The yeast cells in palmwine are predominantly *Saccharomyces cerevisiae* (Bassir, 1968; Faparusi and Bassir, 1972; Owusu, 1987). However, the number of isolates associated with palmwine appear to differ from palmwine to palmwine. Owusu (1987) obtained five isolates from a palmwine specimen, Godwyll and Oduro (unpublished data) reported four isolates while Okraku-Ofei (1968) and Brown (1990) each report only one isolate in their studies covering a number of palmwine samples.

In addition to *cerevisiae*, there have been reports of other *Saccharomyces* species being isolated from fermenting palm juices. Guilliermond (1914) found *Saccharomyces chevalieri* from palmwine of *Elaies guineensis.* Van Pee and Swings (1971) reported the isolation of *Saccharomyces chevalieri, S. pastorianus* and *S.ellipsoides.* Okafor (1972) reported the isolation of *Saccharomyces markii, S. exiguus, S. florentius, S. vafer* and *S. rosei.* Fahwehinmi (1981) also reported the isolation of *S. chevalieri* . All these species are, with the exception of *S. exiguus*, now reduced to synonymy with *S. cerevisiae* Meyen ex Hansen, which has 84 species whose names are regarded as synonyms to *S. cerevisiae* Meyen ex Hansen (Yarrow, 1987). Only seven species are accepted in the genus *Saccharomyces* Meyen ex Hansen (Yarrow, 1987). This has come about as a result of the instability of the characteristics by which they were separated and the absence of reproducible isolation.

Other genera of yeasts have also been isolated from fermenting palm juices. These include *Schizosaccharomyces pombe* (Saito and Otani, 1936; Ahmad *et al.*, 1954), *Endomycopsis fibuliger* (Okafor, 1972), *Candida* sp. (Ahmad *et al.*, 1954; Okafor, 1972), *Pichia* sp. (Fahwehinmi, 1981) and *Hansenula* sp. (Ahmad *et al.*, 1954). Indeed, the literature is replete with diverse claims on the species composition of palmwine dredge or juice. Among the important reasons which may account for the diverse claims on the species composition of palmwine yeast, one may emphasize the fact that the so called different yeasts were all identified on the rather unreliable basis of phenotypic discontinuity.

In Ghana, the widely practised method of tapping palmwine from the felled palm is entirely destructive to the crop. In places where the standing palm is tapped, damage is done to the soft tissues around the point of tapping and this may kill the palm or provide entry for injurious insects, bacteria or other organisms. Tapping also reduces the yield of fruits. For experiments aimed at quality standardisation of large-scale production and preservation of palmwine, these methods are unreliable. Moreover, the identity of the yeasts partly responsible for the varying flavours of palmwine must be clearly established. As yet, little seems to be known about Ghanaian palmwine yeasts. This study was undertaken to examine and identify the palmwine yeasts present in various localities in the southern part of Ghana

The work involved (a) the comparative study of the genomic DNA of palmwine yeasts and (b) a build-up of a partial restriction map of their genomic DNA. The results are expected to confirm or disprove earlier reports on the multiplicity of isolates and the different species, especially, *Saccharomyces* yeasts recorded as being present in palmwine. It is also expected that the current studies will aid in the isolation of economically suitable strains for further work.

1.2 LITERATURE REVIEW

1.2.1 PALMWINE

Palmwine is the fermented palm sap obtained by tapping a palm tree. It is a refreshing alcoholic drink which is popular throughout the warm parts of the world. About 2,500 years ago, people in India, Sri Lanka and other parts of Asia drank both fermented palm and distilled palmwine (Van Pee and Swings, 1971)

Fermented palm sap has been known to the people of the forest belt of West Africa for many years as a refreshing beverage and a drink to be used in traditional ceremonies (Sodah and Matthew, 1971). In Ghana, the fermented sap and the distilled product are equally important, although their relative importance varies from region to region. In areas where crop farming is of paramount importance to the farmer, and where oil palms are not well utilized for the production of oil, a relatively large palmwine industry exists (Sodah and Matthew, 1971). The palmwine industry serves as a substantial source of income to the farmers who tap the trees for the wine.

Palmwine is obtained from two main genera of the palm tree, ***Elaies*** and ***Raphia***.. The species which are most productive are ***E. guineensis***, ***R. vinifera*** and ***R. hookeri***. In Ghana, ***E. guineensis*** is the principal source of sap (Okraku-Ofei, 1968). The quality of the wine, as indicated by its taste, is highly variable and depends, among other factors, on the genus of palm from which the sap is obtained (Okafor, 1972). Within the same palm type, the method of tapping apparently affects the composition of the sap and hence the quality of the wine (Sodah and Matthew, 1971; Okafor, 1972).

There are two methods of tapping the sap (i) from live palms or (ii) from felled trees. In method (i) as practised in Nigeria, Benin and Ivory Coast, the sap is obtained from either the immature male inflorescence (inflorescence tapping) or the stem of a living and standing tree (stem tapping) (Okafor, 1972; Hartley, 1984). In inflorescence tapping, the leaf subtending on an immature male inflorescence is removed to obtain access to the inflorescence enclosed in its spathes. An incision is made near the apex of the inflorescence and the top of the tissues inside the spathes is removed. A piece of the front spathe is removed and the main stem of the spadix is cut horizontally to form a "tapping panel" (Hartley, 1984). The cut is covered with a piece of felt composed of the fibrous leaf sheath fabric and a new slice is taken daily until the wine begins to flow. A funnel of bamboo is inserted in the felt cover which is then set in position and the wine allowed to flow into calabashes or bottles. It is collected in the morning and evening and a new slice is taken from the tapping panel at each collection. In stem tapping, the incision is made on the stem a little below the terminal bud, after the clearing of the older palm fronds so as to expose the point of tapping. The method of collection is the same as for inflorescence tapping (Faparusi and Bassir, 1972; Okafor, 1972).

In method (ii), which leads to the production of the so-called "down-wine" preferred in Ghana, the young trees are felled by cutting the roots and older trees by cutting the trunks one or two feet above the ground (Hartley, 1984). After one or two weeks, when the felled trees have withered to some extent, the fronds in the meristematic region are removed and a rectangular well about 7.5 cm deep is made in the vegetative core. In the floor of the well a round hole is bored through the core into which is inserted a bamboo tube to deliver the sap to the receiver (Hartley, 1984). Frequently, a precautionary measure of "firing" the well is practised in order to reduce the infestation of the well by insects and their larvae, bacteria, moulds and yeasts.

The juice that escapes from an incision of a palm tree or its inflorescence is a sweet and colourless sap containing 4.29 ± 1.4% sucrose, 3.31 ± 0.95% glucose, 0.38 ± 0.015% NH_3 and small amounts of lactic acid and amino acids (Bassir, 1968). The composition of fresh sap from down wine appears to be different from male inflorescence sap as the former is reported to contain glucose, sucrose, fructose, maltose and raffinose (Sodah and Matthew, 1971). A fresh sap contains no alcohol; but after a few hours the production of alcohol usually begins with a rapid evolution of carbon dioxide. The sap is inoculated spontaneously by yeast cells which accumulate in millions in exudates on the flower stalk. These fungi have been shown to be mostly *Saccharomyces cerevisiae* (Bassir, 1968).

Palm sap may be contaminated with bacteria as it drops from the incision. The bacteria most commonly found in fresh wine are *Lactobacillus plantarum* and *Leuconostoc mesenteroides,* and an unidentified *Micrococcus* species (Bassir, 1968). The initial population of microflora usually contains a higher proportion of bacteria; but as the pH of the medium falls to less than 5.5, the yeasts become very active in growth. Besides the chemical composition of the unfermented sap, the nature of the yeasts and other microorganisms contained in palmwine also influence wine quality (Okafor, 1972).

Fresh sap is converted into palmwine in two stages (Bassir, 1962, 1968). The first consists of the production of organic acid by the metabolism of bacteria and the consequent lowering of pH of the juice from 7.4 to 6.8 (Bassir, 1962, 1968). The second stage is the inversion of sucrose by yeast and the production of alcohol, and more organic acid. The second stage is set when the pH of the medium falls to 6.8 and virtually comes to an end when the pH falls to 4.0 (Bassir, 1962, 1968). The whole process usually lasts about 48 hours. Palmwine is usually drunk after about 8 hours fermentation.

Inflorescence wine is reported to contain only ethanol whereas down-wine contains some methanol and propanol (Sodah and Matthew, 1971). Palmwine forms a nutritious drink which provides an important source of vitamin B complex. In Zaire, inflorescence wine was found to contain 7 organic acids, 25 amino acids and vitamin B_{12} (Van Pee and Swings, 1971). Similar wines in Nigeria contained acetic, lactic and tartaric acids as well as 13 amino acids and the vitamins B_1 , B_2, B_6 and C (Bassir, 1962).

1.2.2 YEASTS (GENERAL CLASSIFICATION)

Although the term yeast is used extensively in scientific literature, it does not represent a taxonomic designation that can be rigorously defined (MacMillan and Phaff, 1978). Historically, the word originated from ancient words describing the visible changes occurring in fermenting liquids. As the years passed, other organisms were discovered that were similar but not identical in morphology and physiological properties; and the definition for yeast was expanded to include them, although they were not fermentative.

According to Lodder (1970), "Yeasts may be defined as microorganisms in which the unicellular form is conspicuous and which belong to the fungi", while according to Kreger-van Rij (Kreger-van Rij, 1987), "A yeast may be defined as a unicellular fungus reproducing by budding and fission" These simple definitions are, perhaps, the only ones possible in view of the heterogeneous nature of this group of organisms.

The yeasts are taxonomically diverse and are classified under the Division Eumycota of fungi; under this division three classes of yeasts, namely the Ascomycotina (ascomycetes), the Basidiomycotina (basidiomycetes) and the Deuteromycotina (fungi imperfecti) are recognized (Kreger-van Rij, 1987).

(i) YEAST MORPHOLOGY

Yeasts may differ in their cellular morphology and, to a considerable extent, the morphology exhibited by a particular yeast is directly associated with the mechanism it employs for asexual reproduction (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). Based on morphological features, many kinds of yeast cells have been identified. Cells may be spheroidal, subglobose, ellipsoidal, ovoid, obovoid, cylindrical, botuliform, elongate, filamentous, apiculate, ogival, lunate or triangular (van der Walt and Yarrow, 1987). The cell shape may also be characteristic of a particular genus or species, for example, the lemon-shaped cells of the apiculate yeasts, the bottle-shaped cells of *Malassezia*, the lunate cells of *Metschnikowia lunata* and *Candida pellata* and the triangular cells of *Trigonopsis* (van der Walt and Yarrow, 1987). However, in some yeasts, for example *Saccharomyces cerevisiae*, both the shape and size of cells of different strains of a species are liable to variation (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). Consequently, over-reliance on cell shape or size for yeast identification can be misleading.

(ii) VEGETATIVE REPRODUCTION

Although the majority of yeasts reproduce by budding, fission occurs in some, and in others there is a combination of the two processes (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). Buds may arise either on yeast cells or on hyphae. Depending on how the bud is formed in terms of the ultrastructure of the cell wall, budding may be either holoblastic or enteroblastic. Holoblastic budding is considered to be characteristic of the Saccharomycetales and related anamorphic states, whilst enteroblastic budding is characteristic of basidiomycetous yeasts and related anamorphic states (van der Walt and Yarrow, 1987).

Budding may also be described in terms of the position of the budding sites. If budding is restricted to the pole of the mother cell, it is referred to as monopolar budding and may occur on a rather broad base, eg. in *Malassezia* (van der Walt and Yarrow, 1987). If buds are formed exclusively at the distal poles of the mother cell, it is referred to as bipolar budding. Bipolar budding is characteristic of the apiculate yeasts. Multilateral or multipolar budding implies budding at different sites on the mother cell. If a yeast reproduces exclusively by budding, the mature bud may either detach itself immediately or remain attached to the mother cell and eventually give rise to either clusters of cells or chains of cells. The tendency of some yeasts to form chains of cells, results in the formation of pseudohyphae or pseudomycelium.

Reproduction by fission implies the duplication of a vegetative cell by means of the ingrowth from cell wall of a transverse septum which bisects the long axis of the cell. The newly formed fission cells, which are arthroconidia (arthrospores), elongate, and the process repeats itself (van der Walt and Yarrow, 1987). Reproduction by fission is characteristic of the genus *Schizosaccharomyces* (van der Walt and Yarrow, 1987).

Vegetative reproduction by formation of conidia borne on stalk-like, tubular structures is relatively rare among the yeasts (van der Walt and Yarrow, 1987). This mode of reproduction entails the formation by a mother cell of one or more tubular protuberances each of which gives rise to a single terminal conidium. After maturation, the conidium is disjointed at the septum in the mid-region of the protuberance. The conidia are not discharged. This mode of reproduction is diagnostic of the genus *Sterigmatomyces* (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987).

The formation of discharged spores or ballistospores (ballisto conidia) is a specialized mode of reproduction which is characteristic of certain basidiomycetous genera, eg. *Sporobolomyces* (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). When agar plates containing colonies of these yeasts are inverted for a short time over fresh plates, spores are forcibly discharged onto the lower plates. Upon germination, these form new colonies that are "mirror images" of the original ones (MacMillan and Phaff, 1978).

Some yeast species such as members of the genus *Metschnikowia* and *Candida albicans* form chlamydospores (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). The chlamydospore has been defined as a thick walled, non-deciduous, intercalary or terminal, asexual spore formed by the rounding of a cell or cells (Ainsworth, 1971). Mature chlamydospores have particular affinities for certain dyes.

The formation of asexual endospores is not a common phenomenon but has been observed in the genera *Trichosporon, Candida, Cryptococcus* and *Oosporidium* (van der Walt and Yarrow, 1987). These endospores are vegetative cells which are delimited within the cells or hyphae. The cells, unlike chlamydospores or ascospores cannot be stained selectively.

(iii) CULTURAL CHARACTERISTICS

The cultural characteristics of yeasts, on solid or in liquid media, are sometimes sufficiently unique to be of taxonomic value (MacMillan and Phaff, 1978).

Distinctive growth on such solid media, as malt agar, may be a manifestation of hyphal or pseudohyphal growth. On solid media, the texture, colour, surface, margin and elevation of the streaked culture are noted. The texture may be mucoid, butyrous, friable, coherent or tenacious. Mucoid growth is frequently associated with encapsulation of cells, as a result of the production of extracellular polysaccharide material, while matted, coherent growth is generally associated with the formation of an abundance of pseudohyphae or true hyphae (van der Walt and Yarrow, 1987). Some yeast species are also characterized by the production of distinctive colours (van der Walt and Yarrow, 1987; Davenport, 1980). The presence of yellow, orange and red carotenoid pigments is, for instance, characteristic of the genera *Rhodotorula, Sporobolomyces, Phaffia, Rhodosporidium* and *Sporidiobolus*, while the production of the non-carotenoid, Bordeaux-red pigment, pulcherrimin, is typical of only certain yeasts, eg. *Metschnikowia pulcherrinma* and certain *Kluyveromyces* species (van der Walt and Yarrow, 1987). The majority of yeasts, however, produce colonies ranging from white to cream or light shades of brown.

The peculiar surface of colonies on solid media may also be used to classify yeasts. Colony surfaces may be glistering or dull, smooth, sectored, striated, pulvinate, verrucose, plicate, rough or hirsute (van der Walt and Yarrow, 1987). For example, in

Saccharomyces species, the growth surface of colonies is often semi-glossy (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). Some yeasts are, however, characterized by both rough and smooth forms of growth, eg. *Candida albicans* and *Trichosporon cutaneum (beigelii)* (van der Walt and Yarrow, 1987). The elevation, that is, whether the growth is flat and spreading, or raised and restricted, and the margin, that is, whether the edge of the streak culture is smooth or entire, undulating, lobate, rhizoid,- erose or fringed with pseudohyphae or hyphae, are all important.

Yeast growth in liquid media may result in the formation of a compact, coherent, flocculent or mucoid sediment, a ring, islets or a pellicle properties that are readily identifiable and of some value in yeast species characterisation (MacMillan and Phaff, 1978). The development of any characteristic smell, from such compounds as esters, is also important.

(iv) SEXUAL CHARACTERISTICS

Many yeasts are characterized by sexual reproduction involving an alternation of generations with the formation of characteristic cells in which meiosis occurs. In the ascogenous yeasts, the site of meiosis is the ascus in which the haploid generation or ascospores are delimited internally. In basidiomycetous yeasts, meiosis is restricted to the basidium in which the haplophase is externally delimited as basidiospores (van der Walt and Yarrow, 1987). If a yeast is characterized by the formation of either asci or basidia, it is referred to as a perfect yeast or a perfect (teleomorphic) state. Yeasts which lack sexual stages, are termed imperfect yeasts or imperfect (anamorphic) states (Hennebet and Weresub, 1977).

(a) Ascomycetous yeasts

Yeasts that produce ascospores are either homothallic (fusing nuclei are identical) or heterothallic (fusing nuclei are not identical). Their life cycles are further characterized on the basis of the ploidy of the vegetative reproductive stage, which is either haploid or diploid or a mixture of the two phases (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987).

In the case of homothallic yeasts which are vegetatively stabilized exclusively in the haplophase, plasmogamy, karyogamy and meiosis occur within the zygote which is, as a rule, constituted by the fusion of two vegetative cells. The diplophase is shortlived and restricted to the diploid condition of the zygote (Kreger-van Rij, 1987). In the case of the homothallic yeast strains, which are vegetatively stabilized in the diplophase, a single diploid cell may undergo reduction division and be converted directly into an unconjugated ascus, eg. the genus *Saccharomyces*. In such a cycle, the haploid condition is short-lived and restricted to the ascosporal stage.

In the case of heterothallic yeasts, the diplophase is normally heterozygous for mating type genes and individual cells are bisexual. The existence of unisexual diploid material has also been reported (Wickerham, 1958). If the diplophase is stabilized, the asci remain unconjugated and unisexual haploid ascospores of both mating types are formed. Two ascospores conjugate directly in the ascus, and the first bud from this zygote is a diploid eg. *Saccharomycoides ludwigii*, or ascospores may germinate and bud as haploid cells for a short time prior to conjugation, eg. *Saccharomyces cerevisiae* (van der Walt and Yarrow, 1987).

In certain species, eg. *Pichia membranae faciens* and *Pichia spartinae*, strains may be either homothallic or heterothallic (van der Walt and Yarrow, 1987). Various yeasts cannot also be categorized as strictly haploid or diploid; and in some yeast cultures, both haploid and diploid vegetative cells may exist side by side.

The form of the ascus can be characteristic of a genus, eg. large clavate, ellipsoidopedunculate asci are typical of *Metschnikowia*, while the crowned asci of *Stephanoascus* readily differentiate this genus from others among the filamentous yeasts (van der Walt and Yarrow, 1987).

Ascospores delimited within the ascus may vary from 1-4, eg. in *Lodderomyces*, *Schwanniomyces* and *Saccharomyces*, or 8-16, as in *Lipomyces* and *Kluyveromyces* (van der Walt and Yarrow, 1987; Fowell, 1969). By comparison, *Dipodacus* produces an indefinite number of ascospores (up to 100) (MacMillan and Phaff, 1978), and one species, *Kluyveromyces polysporus* up to 1000 (Fowell, 1969). Considerable variation in the shape of ascospores is encountered among different yeast species, as ascospores may be spheroidal, ellipsoidal, clavate, lentiform, saturniform or galeate. Spore morphology is a fairly distinct property which is useful in species identification. Thus, cup-shaped ascospores are characteristic of the genus *Wickerhamia*, spindle-shaped ascospores of the genera *Ambrosiozyma*, *Dekkera* and some species of the genera *Hansenula* and *Pichia* (van der Walt and Yarrow, 1987). In some yeasts, eg. *Pichia ohmeri*, some variation in ascosporal shape may be observed within the same species. In certain genera, eg. *Lipomyces*, *Pichia*, *Hansenula* and *Wingea*, ascospores may be pigmented with the result that actively sporulating material assumes an amber, brown or reddish-brown colour (van der Walt and Yarrow, 1987).

(b) Basidiomycetous yeasts

The basidiomycetous yeasts occur either as the budding haplophase, the dikaryotic mycelial phase or the self-sporulating diplophase.

Sexual reproduction in the basidiomycetous yeasts may be either heterothallic or homothallic. In the heterothallic species, the dikaryotic mycelium is produced by one of the conjugants after mating of a pair of compatible cells. The dikaryotic mycelium ultimately forms clamped cells in which karyogamy occurs. These cells may be intercalary, terminal or lateral, and may be thick-walled or not. In the homothallic or self-fertile strains, homothallism is either primary or secondary. In strains having primary homothallism the mycelium is uninucleate and does not exhibit clamp formation; the mycelium of strains with secondary homothallism is dikaryotic and exhibits clamp formation (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987).

In the ustilaginaceous genera, *Rhodosporidium* and *Leucosporidium*, the thick-walled cells have been referred to as teliospores, teleutospores and ustilospores (van der Walt and Yarrow, 1987). These spores may vary in shape, ranging from spheroidal to ovoidal and angular, and may be markedly pigmented as in the genus *Rhodosporidium* (van der Walt and Yarrow, 1987). Teliospores are produced either terminally or within the hyphal strands, and karyogamy takes place. Eventually the teliospores germinate, with the formation of a promycelium or germ tube. Reduction division occurs and the promycelium becomes septate, forming four cells on which sporidia (basidiospores) are borne. Segregation into original mating types occurs during the formation of sporidia. Sporidia can reproduce as budding yeast cells and can conjugate and repeat the cycle. (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). In *Leucosporidium scottii* the diploid nucleus migrates into the promycelium where it undergoes reduction division, and the four haploid nuclei distribute themselves within the promycelium (van der Walt and Yarrow, 1987). In *Rhodosporidium toruloides*, however, meiosis occurs within the teliospore itself and the four haploid cells migrate into the promycelium (van der Walt and Yarrow, 1987). In some other species, eg. *Leucosporidium gelidum*, the promycelium does not become septate and the sporidia develop terminally (van der Walt and Yarrow, 1987).

(c) Imperfect yeasts

This group of the fungi imperfecti includes yeasts for which the perfect state has not been described. This state may include either asci or basidia. The yeasts may be heterothallic haploids for which no mating types have been found, or they may be haploid or diploid yeasts with unknown conditions for sporulation (Kreger-van Rij, 1987).

Several imperfect yeasts closely resemble the perfect yeasts in morphological, physiological and biochemical properties and, for that reason, are considered to be the imperfect state of the perfect species. For most imperfect species, the relationship to the perfect state is unknown; they may belong to one of the described perfect genera or to undescribed genera (Kreger-van Rij, 1987).

Classification of the imperfect yeast is, in the absence of the taxonomically important characters of sexual reproduction, defective in that a genus may include the imperfect state of species of several genera (Kreger-van Rij, 1987). Differentiation of imperfect species and genera that are analogous to the perfect species and genera is often impossible because suitable distinguishing characters are lacking.

(v) PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

Physiological properties primarily serve to describe, differentiate and identify yeast strains. They may also serve to describe, characterize, and differentiate species, and to a lesser extent, genera (van der Walt and Yarrow, 1987). The properties most commonly used are those related to the utilisation of carbon and nitrogen sources, growth factor requirements, growth at elevated temperatures and on media with high sugar or sodium chloride content, the formation of typical characteristic metabolites and the susceptibility of the yeast to antibiotics (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). Recently, criteria for delimiting relationships among various yeasts have been based on immunological properties, analysis of the base composition of deoxyribonucleic acid, cell ultrastructure, the chemical composition of the cell and the repertoire of enzymes found in the cell (MacMillan and Phaff, 1978; Kreger -van Rij, 1987).

(a) Utilisation of carbon and nitrogen compounds

Although yeasts, generally, have the ability to ferment various sugars, this ability to ferment the sugars may vary from one genus to another. For example, the genera *Kluyveromyces*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces* are all characterized by vigorous fermentation of at least glucose, while other genera, such as *Lipomyces* and *Sterigmatomyces* do not ferment sugars at all; and lying between these two extremes, are other genera, eg. the *Hansenula*, in which are found an entire range of cells, from nonfermentative to strongly fermentative species (van der Walt and Yarrow, 1987). If fermentation occurs, glucose will always be fermented. For identification purposes, the ability to ferment glucose, galactose, sucrose, maltose, lactose and raffinose is routinely examined. The ability to ferment a specific sugar is, however, not regarded as stable and immutable (van der Walt and Yarrow, 1987). The same carbon sources that a yeast can ferment can also be assimilated oxidatively under appropriate conditions. The reverse does, however, not hold (MacMillan and Phaff, 1978; van der Walt and Yarrow, 1987). About 18 or more specific carbon compounds have been described for carbon assimilation tests (Wickerham and Burton, 1948; Wickerham, 1951; van der Walt and Yarrow, 1987). The taxonomic value of fermentation reactions is, generally, considered to be lower than that of assimilation reactions (Kreger-van Rij, 1987).

Yeasts are capable of utilizing a diversity of nitrogen compounds. The ability or inability to utilize nitrate-nitrogen, as a diagnostic criterion, is particularly valuable for determinative purposes. Many genera, such as *Saccharomyces* and *Kluyveromyces*, are characterized by their inability to utilize nitrates, while in other genera, eg. *Hansenula* and *Pachysolen*, all species utilize nitrate (van der Walt and Yarrow, 1987). Among the imperfect genera, eg. in *Candida* and *Trichosporon*, both nitrate positive and nitrate negative species occur. With a few exceptions, the utilisation of nitrate, at least within the species, is stable (van der Walt and Yarrow, 1987). For identification purposes, the utilisation of nitrate, nitrite, ethylamine hydrochloride, cadaverine hydrochloride, L-lysine, creatinine or creatine as sole source of nitrogen, has been found to be most useful (Wickerham, 1946; van der Walt and Yarrow, 1987).

(b) Growth factor requirements

Different yeasts have extremely divergent demands with respect to their growth factors. The use of the diagnostic property based on the ability or inability to grow in a mineral medium devoid of vitamins, was introduced by Wickerham (1951). The most common vitamins that have been generally adopted include biotin, pantothenate, folic acid, m-inositol, niacin, p-aminobenzoic acid, pyridoxine, riboflavin and thiamine (van der Walt and Yarrow, 1987).

The ability to grow in a vitamin-free medium varies for different yeasts. Strains of the genera *Hanseniaspora* and *Kloeckera* for example, have been found to have an absolute requirement for m-inositol and pantothenic acid, while *Dekkera*, *Metschnikowia* and *Brettanomyces* species require biotin and thiamine (van der Walt and Yarrow, 1987). Some yeast species may also vary in their vitamin requirements. For example, only a few yeast strains need p-aminobenzoic acid for growth; they include *Rhodotorula* (Suomalainen and Oura, 1969) and some strains of brewers top yeasts (Rainbow, 1948).

(c) Growth at elevated temperatures

Growth, sporulation and survival of yeasts are intimately related to ambient temperature. Temperatures between 20 and 28°C favour the growth of most yeast species. There are, however, exceptions, particularly those species which are restricted to very specific habitats. Psychrophiles, which are common in bodies of cold water, proliferate very poorly at 20°C but grow well at 15°C or 4°C. On the other hand, psychophobic species such as *Saccharomycopsis guttulata*, *Candida sloofii* and *Torulopsis pintolopesii*, which are able to grow only within a narrow range of temperatures (with 20-28°C as the lower limit and 42-45°C as the upper limit), are adapted to life in the digestive tract of warm-blooded animals (Carmo-Sousa, 1969). The maximum temperature of growth has been shown to be a valuable species characteristic (Vidal-Leira *et al.*, 1979).

(d) Growth in media of high osmotic pressure

Growth in media of high osmotic pressure (50% glucose and NaCl + 5% glucose) varies for many yeast species. Yeasts recovered from substrates with high sugar or salt content are generally resistant to high osmotic pressure, eg. some *Schizosaccharomyces* species isolated from high sugar environments (van der Walt and Yarrow, 1987). Few yeast species are capable of development with sugar concentrations of between 50-70%, although a large variety of species can tolerate glucose concentrations of up to 40% by weight (van der Walt and Yarrow, 1987).

(e) Formation of typical characteristic metabolites

The use of the formation of typical metabolites such as acid production from glucose, splitting of arbutin, production of extracellular amyloid compounds, production of ammonia from urea, fat splitting, ester production, gelatin liquefaction and pigment formation have been found to have limited application as diagnostic criteria (van der Walt and Yarrow, 1987). Most, if not all, yeast cultures produce traces of volatile and nonvolatile acids and it is only when excessive amounts of, say, acetic acid are produced that acid formation can be of diagnostic use. Under similar cultural conditions, several encapsulated yeast strains have been found to form extracellular polysaccharides which give a blue or greenish-blue colour with iodine solution (Aschner *et al.,* 1945). Investigations on the hydrolysis of urea by a number of ascogenous and unascogenous species on Christensen's urea agar (Christensen, 1946) showed urease activity is generally lacking in the ascogenous species, whereas it is particularly marked in the basidiomycetous genera *Cryptococcus* and *Rhodotorula* (Seeliger, 1956). Ester production also has limited application since sufficient amounts need to be produced to be easily detected by smell, before this property can be used for the characterisation of yeast species.

(f) Susceptibility to antibiotics

Yeasts differ in their sensitivity towards the antibiotic actidione (cycloheximide) and can be divided into three categories on the basis of this property. For example, some species such as *Saccharomyces cerevisiae* are markedly sensitive (inhibited by 1µg/ml); others such as *Saccharomyces pombe* are moderately sensitive (inhibited by 25 µg/ml), while there are those species, such as *Kluyveromyces lactis* which are not inhibited by concentrations as high as 1000 µ g/ml (Whiffen, 1948).

Because of the possibility that strains may become adapted to low concentrations of this antibiotic, the resistance to this compound finds limited application in the characterisation of species when tested in liquid medium at concentrations of 100 ppm and 1000 ppm (van der Walt , 1970).

(g) Immunological properties

The yeast cell is a traditional source of complex antigenic compounds which tend to generate antisera with wide ranges of antibodies (Streiblova, 1988). Analysis, using the slide agglutination method, of the antigenic structure of yeast species of several genera has been made (Tsuchiya *et al.,* 1965). Both thermostable and thermolabile antigens were found. The results obtained were found useful for the systematics of yeasts, especially, for the differentiation of genera. For example, species in the genus *Hansenula*, which seems to be very homogenous, appeared to be uniform in antigenic structure. On the otherhand, failure to find mutual antigens in species, a situation which was found in *Schizosaccharomyces pombe, Candida albicans* and *Cryptococcus neoformans,* confirms the very separate taxonomic position of these species. These obvious correlations between the antigenic structure and other taxonomic criteria make it worth considering a relationship between different genera when a striking resemblance in the antigenic structure exists, as for instance, between *Debaryomyces hansenii,- Citeromyces matritenis* and *Schwanniomyces occidentalis*. However, resemblances in antigenic structure may be found which do not correlate at all with other features of these yeasts.

An electrophoretic analysis of the antigens of several yeast species has also been made (Biguet *et al.,* 1965). The electrophoretic analysis, in contrast with Tsuchiya's method which demonstrated the antigens of the cell wall, which were generally insoluble (Tsuchiya *et al.,* 1965), revealed the presence of soluble antigens of the whole cell which were precipitated by antibodies. Similar results were obtained with these two complementary methods. A great resemblance was found in the immunoelectrophoretograms of strains presumed to be the perfect and imperfect forms of species, for instance, *Hansenula anomala* and *Candida pelliculosa.*

(h) Ultrastructure and chemical composition of the cell wall

All yeasts have a cell wall. The yeast cell wall is an envelope consisting of intermeshed polysaccharide microfibrils embedded in a complex matrix composed of various polysaccharides, proteins and lipids (Bartnicki-Garcia and McMurray, 1969). The polysaccharide components of the yeast cell wall are glucans, mannans and chitin (Carmo-Sousa, 1969). The yeast cell wall is stratified and its thickness, which varies from species to species, is thinnest in young cells (Kreger-van Rij, 1987). In transmission electron microscopy (TEM) of sections of suitably fixed cells, the walls show electron-light and electron-dense layers. Structural details of taxonomic importance may be observed in the lateral wall of vegetative cells, the hyphal septum and the ascospore wall (Kreger-van Rij, 1987).

A distinct difference has been observed between the wall of ascomycetous and basidiomycetous yeasts (Kreger-van Rij and Veenhuis, 1970); the wall of ascomycetous yeasts, in sections of fixed material, has a broad light inner layer and a thinner dark outer layer; basidiomycetous yeasts have a wall composed of a variable number of thin dark and light layers giving it a lamellar appearance. The imperfect basidiomycetous and ascomycetous yeasts show the same difference in structure and, as a consequence, the ultrastructure of the cell wall may be used to recognize a non-sporulating yeast as ascomycetous or basidiomycetous.

The chemical composition of the yeast cell wall can also be used as a basis for differentiating yeasts (Carmo-Sousa, 1969; Phaff, 1971). The composition of polysaccharides that make up the cell wall provides such information. There is evidence, from enzymatic studies that shows there are quantitative differences (Cutley, 1988) and structural differences in the various polysaccharides (Kreger-van Rij, 1987).

Typical components of the cell wall carbohydrates are the β-1,3- and β-1,6-D-glucans, α-1,3- and α-1,4-D-glucans, cellulose, mannoproteins and chitin (Cabib *et al.*, 1982). There is evidence that these populations of polymers may be covalently linked to one another; for example chitin to protein and β-D-glucan (Sietsma and Wessels, 1979), and β-D-glucan to mannoprotein (Elorza *et al.*, 1985). Very significant differences have been demonstrated in the carbohydrate moiety, particularly in the side chains of the branched mannan molecule (Kreger-van Rij, 1987).

The chitin content of the cell has also been found to differ among the yeasts. Budding ascomycetous yeasts contain only a small amount of chitin amounting to 1-2% of the cell wall on dry weight basis. The chitin content of the walls of filamentous ascomycetous yeasts appears to be significantly higher (Kreger-van Rij, 1987). The cell walls of the fission yeasts, on the other hand, lack chitin.

(i) DNA Composition and Relatedness

A knowledge of the overall DNA base composition of organisms is a valuable preliminary tool for assessing their relatedness. Organisms closely related at the species level would be expected to have similar, or nearly similar base compositions; those organisms which are unrelated need not have any comparable DNA base compositions. The importance of DNA base analysis as a taxonomic aid for yeasts was recognized after development in bacterial systematics, resulting from comparisons of the prokaryotic genome (Marmur *et al.,* 1963).

Nucleic acid comparisons among yeasts were originally limited to DNA base composition determinations (Nakase and Komagata, 1970a, 1970b; Martini *et al.,* 1972). Thermal denaturation (Marmur and Doty, 1962) and buoyant density properties of nucleic acids in cesium density gradients established by ultracentrifugation (Schildkraut *et al.,* 1962) have been used to determine base composition of nuclear DNA. However, determination of G+C content, obtained from thermal denaturation and ultracentrifugation, cannot distinguish between the relationships among yeasts with similar DNA base pair compositions (Kreger-van Rij, 1987). Subsequently, DNA-DNA complementarity analysis was developed to quantitate the similarity of convergent yeasts, using several methods to examine DNA sequence relatedness among yeasts (van der Walt , 1980).

DNA-DNA homology experiments have proved to be the most valuable for species delimitation (Price *et al.,* 1978). Low DNA-DNA homology between species correlated with lack of interfertility among yeast strains (van der Walt , 1980), although the lower limits of DNA-DNA homology values, suggesting species delimitation, were not well defined (van der Walt , 1980). Generally, however, species showing a very low degree of DNA homology have been considered to be different. High DNA-DNA homologies have been shown to indicate conspecificity (van der Walt , 1980).

(j) Similarity of Enzymes

Two organisms differing in the DNA base composition or sequence, or both, would code for an enzyme which might catalyze similar reactions, but would be structurally unrelated. For the analysis of specific enzymes from related organisms to represent a significant evaluation of an essential taxonomic character, it would have to be demonstrated that the enzymes have been coded by genetically homologous, structural genes (Marmur *et al.,* 1963).

Taxonomy by allozyme analysis is based on the premise that genetic diversification is a function of time. This means that organisms that are recently separated should show a high degree of similarity among macromolecules and those more distantly separated should exhibit an increasing number of differences in their DNA and proteins. Allozyme analysis as a technique is much more expressive of intraspecific variation than DNA-DNA sequence complementarity (Kreger-van Rij, 1987).

Many methods have been used for comparing enzymes, for example amino acid composition, amino acid sequences, fingerprint patterns, electrophoretic properties and chromatography. The ones most useful to microbial taxonomists are those which compare enzymes, isolated from different organisms with respect to their catalytic, immunological, or physical properties (Marmur *et al.,* 1963).

For yeasts, coenzyme Q analysis (Yamada *et al.,* 1973) has revealed that the coenzymes in yeast vary from Q-6 to Q-10. In many genera, all species have the same Q system, although a few exceptions occur in some genera, such as *Pichia* and *Hansenula* (Yamada *et al.,* 1973). Studies on the coenzyme Q system of ascosporous yeasts have been useful in correlating the taxonomic relationship of imperfect yeasts with certain groups of perfect genera (Kreger-van Rij, 1987).

Baptist and Kurtzman (1976) utilized comparative enzyme patterns to separate sexually active strains of *Cryptococcus laurentii* var *laurentii* from nonreactive strains and from the varieties *magnus* and *flavescens.*

1.2.3. MOLECULAR GENETIC ANALYSIS

The molecular genetic approach to yeast speciation has its origin in modern developments in bacterial systematics. This has resulted from the many initial studies that have made possible chemical and biochemical manipulations of DNA.

Although yeasts have greater genetic complexity than bacteria, they share many of the technical advantages that permitted rapid progress in the molecular genetics of prokaryotes and their viruses. The interest in studying yeast has been greatly spurred by the realisation that, as eukaryotes, yeast cells are organized much like cells in more complex organisms.

(i) THE YEAST GENOME

Yeasts have well defined genetic and highly versatile DNA transformation systems. Although genetic analyses have been undertaken with a number of taxonomically distinct varieties of yeasts, extensive studies have been restricted primarily to the many freely interbreeding species of the budding yeast *Saccharomyces* and to the fission yeast *Schizosaccharomyces pombe* (Watson *et al.,* 1992).

The *Saccharomyces cerevisiae* yeast genome (1.4 x 10^7 bp) is very small; it is only a few times larger than that of *E.coli* (4 x10^6 bp) and 200-fold smaller than that of mammalian cells (3.5 x 10^9 bp), which greatly simplifies both genetic and molecular analysis (Watson *et al.,* 1992). *Saccharomyces cerevisiae* contains a haploid set of 16 chromosomes which vary in size from 200-2,000 kb, with a total length of 14,000 kb (Sherman, 1991). The chromosomal genome is densely packed with an estimated 6,500 genes having an average size of 2 kb and few introns (Sherman, 1991). In addition, chromosomes contain movable DNA elements, retrotransposons or transposable (Ty) elements, that vary in number and position in different strains of *S. cerevisiae* .

In addition to the normal chromosomal complements of genes, yeasts also possess 2-µm plasmids. These are 6.3 kb circular duplex DNA (Livingston and Hahne, 1979) which exist within the nuclear envelope of most yeast cells as deoxyribonucleoprotein histone complexes (Sherman, 1991).

Other nucleic acid entities can also be considered part of the yeast genome. Mitochondrial DNA encodes components of the mitochondrial translational machinery, and approximately 15% of the mitochondrial proteins (Sherman, 1991). Some mutants completely lack mitochondrial DNA, although they retain mitochondria which are morphologically abnormal (Sherman, 1991). Almost all *S. cerevisiae* strains contain double stranded (ds) RNA viruses that are not normally infective but are transmitted by mating (Sherman, 1991). This ds RNA, constituting approximately 0.1% of total nucleic acids, determines components required for the viral transcription and replication (Sherman, 1991).

Approximately 85% of the sequences in yeast chromosomes are unique and up to 15% of the nuclear DNA consists of repeated sequences (Philippsen *et al.,* 1991). The four classes of repeated DNA (rDNA, 2-µm plasmid, Ty elements, telomeric Y'sequences) are the origin of most bands seen in restriction spectra (Philippsen *et al.,* 1991). The ribosomal DNA consists of a cluster of usually 100 to 200 tandem copies of a 9.08 kb repeat unit (Philippsen *et al.,* 1991). The 2-µm plasmid is present in 50 to 100 copies in many, but not all, *S. cerevisiae* strains. Natural isolates of yeast carry only a few copies of the mobile elements Ty 1, Ty 2, Ty 3 and Ty 4 (Clark *et al.,* 1988). The telomeric Y' sequences are also carried by some natural isolates of *S.cerevisiae* as single copies or short clusters at the ends of their chromosomes (Philippsen *et al.,* 1991).

(ii) RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Polymorphism of nucleotide sequences in the DNA of several organisms has proven useful in distinguishing between strains and determining their relatedness to one another (Patterson and Hyypia, 1985).

Until recently, polymorphisms could be detected only if they were expressed by differences in the behaviour of a protein, for example, by differences in enzymatic activity or electrophoretic mobility. This situation changed dramatically with the realisation that sites recognized by restriction endonucleases could be polymorphic (Watson *et al.,* 1992). This is because mutations would have caused the loss of sites at which a particular restriction enzyme can act. Since these sites are only present in the genome of certain species, they are polymorphic.

Polymorphisms detected in this way are known as restriction fragment length polymorphisms. RFLPs can be used to characterize an organism, levels of genotypic diversity, and phytogenetic relationships. They can arise from point mutations leading to either a loss or a gain of a site at which a restriction endonuclease acts. In addition, deletions or insertions resulting in variations in the number of tandemly repeated DNA sequences can alter the length of fragments between two endonuclease sites (Watson *et* *al.,* 1992). Since DNA polymorphisms can affect any type of DNA sequence, this means that an alteration producing a restriction fragment polymorphism length can occur within a coding sequence of a gene, noncoding sequences (introns), sequences between genes, and even DNA with no known function, such as repetitive DNA. Because the DNA polymorphism can affect any part of the genome and they need not be expressed as a protein product, they are extremely valuable markers (Rothwell, 1988).

If the total genomic DNA is digested to completion with a restriction endonuclease, an extremely large number of fragments will be produced. These can be separated by electrophoresis and will show up as weak or strong bands. When DNA from many species is examined in this way the bands will be found to be different. These restriction spectra will be specific, not only for individual restriction endonucleases but also, for the genomes of the different species (Philippsen *et al.,* 1991).

(iii) ISOLATION OF DNA

One of the first steps in the *in vitro* manipulation of DNA involves the isolation of the DNA. Most DNA *in vivo* is present in association with RNA and proteins. Proteins directly involved in the process of gene expression, such as RNA polymerase regulatory proteins, interact with DNA *in vivo* to form nucleoprotein complexes. DNA polymerase, DNA ligase, various unwinding and supercoiling enzymes, recombination and repair enzymes, and those proteins involved in the initiation or maintenance of DNA replication are also associated with DNA *in vivo* (Rodriguez and Tait, 1983). It is, therefore, necessary first to isolate crude complexes of nucleoproteins from cells and then purify and separate DNA from proteins and RNA. The basic steps involved are (a) the release of soluble, high molecular weight DNA from disrupted cells and membranes (b) dissociation of DNA-protein complexes by denaturation or proteolysis; and (c) the separation of DNA from other macromolecules (Marmur, 1963; Rodriguez and Tait, 1983).

The greatest difficulty in the isolation of highly polymerized DNA from yeast has been to obtain a relatively simple and gentle method of cell lysis (Smith and Halvorson, 1967). Yeast cells can be lysed by mechanical disruption, by the formation of sphaeroplasts following enzymatic degradation of their cell walls and subsequent lysis in a detergent, or by freeze-thawing in sodium dodecyl sulphate (SDS) (Smith and Halvorson, 1967). Since the mechanical disruption of yeasts increases the probability of scission of the isolated DNA (because of its large length-to-width ratio), this method is not often used (Marmur, 1963; Smith and Halvorson, 1967).

After cell lysis, the cell debris and proteins are removed by denaturation and centrifugation. Several methods have been developed for deproteinizing the lysed cell suspension: shaking with a chloroform/isoamyl alcohol mixture (Marmur, 1963), by enzymatic degradation of the proteins with pronase (Hotta and Bassel, 1965), or by shaking with phenol (Kirby, 1968). RNA is removed by RNase (Marmur, 1963; Rodriguez and Tait, 1983). The DNA is selectively precipitated by the addition of ethanol or isopropanol. Precipitation from alcohol serves to concentrate the high molecular weight DNA while removing the small oligonucleotides of DNA and RNA, detergent , and the organic solvents used in the removal of proteins (Rodriguez and Tait, 1983). Degradation by DNase and divalent metal ion contamination is prevented by the presence of chelating agents and by the action of SDS (Marmur, 1963)

Once the DNA preparation has been freed of contaminating macromolecules, the concentration of the DNA in the solution can be determined. The method most commonly used for this purpose is ultraviolet absorption spectroscopy (Rodriguez and Tait, 1983). Ultraviolet absorption spectroscopy is also used to determine the relative purity of the DNA.

(iv) RESTRICTION ENDONUCLEASES

The class of enzymes known as restriction endonucleases have played a key role in the development of recombinant DNA technology. These bacterial enzymes possess an endonuclease activity which is directed to a specific sequence of bases in double-stranded DNA. In nature, they serve to protect bacteria from the possible incorporation of foreign DNA into their genomes by digesting such material. The bacterium's own DNA is protected by being methylated on A or C residues, which renders it unavailable for digestion by its own enzymes (Smith and Nathans, 1973; Hawkins, 1986; Watson *et* *al.,* 1992). It has been suggested that these enzymes may also play a role in promoting " site-specific illegitimate recombination ", allowing incoming DNA to be cleaved and incorporated into the chromosome (Rodriguez and Tait, 1983). The term " restriction" arose because it was originally found that certain bacteriophages would not grow on certain bacterial strains; hence they were said to be restricted (Hawkins, 1986). Investigation of this phenomenon revealed that it was due to the action of this class of enzymes (Arber, 1976).

Restriction enzymes have been classified into three groups. Type I and type III enzymes carry modification (methylation) and ATP-dependent restriction (cleavage) activities in the same protein (Sambrook *et al.,* 1989). Type III enzymes cut the DNA at the recognition site and then dissociate from the substrate. However, type I enzymes bind to the recognition sequence but cleave at random sites when the DNA loops back to the bound enzyme. Neither type I nor type III restriction enzymes are widely used.

Type II restriction/modification systems are binary systems consisting of a restriction endonuclease that cleaves a specific sequence of nucleotides and a separate methylase that modifies the same recognition sequence (Sambrook *et al.,* 1989). These enzymes recognize specific sequences of four to eight base pairs in length (Watson *et al.,* 1992). The sequences in the two strands of DNA that are recognized by the enzymes possess a two-fold axis of symmetry (Rodriguez and Tait, 1983; Hawkins, 1986; Sambrook *et al.,* 1989). The location of cleavage sites within the axis of a dyad symmetry differs from enzyme to enzyme. Some enzymes make cuts which are exactly opposite in the two DNA strands, so that the ends are said to be 'blunt': others cleave each strand at similar locations on opposite sides of the axis of symmetry, creating fragments of DNA that carry protruding single-stranded termini (Rodriguez and Tait, 1983; Hawkins, 1986; Sambrook *et al.,* 1989).

Restriction enzymes that cut specific sequences have been isolated from several hundred bacterial strains, and over 150 different specific cleavage sites have been found (Watson *et al.,* 1992). In other to simplify the naming of these enzymes, a nomenclature has been developed that is based on an abbreviation of the name of the organism from which the enzyme was isolated (Smith and Nathans, 1973; Smith, 1979). The first initial of the genus and the first two initials of the species form the basic name. This may be followed by a strain designation, when the enzyme is present in a specific strain, or a Roman numeral to differentiate enzymes from the same source. For example, *Hae* II is one of the three enzymes purified from the strain *Haemophilus aegypticus* and *Hin*f I is the enzyme purified from *Haemophilus influenzae* strain f (Rodriguez and Tait, 1983). In general, different restriction enzymes recognize different sequences. However, there are many examples of enzymes isolated from different sources that cleave within the same target sequences. These are known as isoschizomers (Rodriguez and Tait, 1983; Hawkins, 1986; Sambrook *et al.,* 1989). An example of such enzymes are, *Hind* III and *Hsu* I (Rodriguez and Tait, 1983).

(v) AGAROSE GEL ELECTROPHORESIS

Electrophoresis, together with restriction enzyme technology, has played an essential part in the analysis of the structure, sequence and function of DNA. Electrophoresis through agarose or polyacrylamide gels is the standard method used to separate, identify and to purify DNA fragments (Sambrook *et al.,* 1989). The method is used for DNA fragments generated after endonuclease digestion, before or after enzymatic modification, ligation with other fragments, or after sequencing (Perbal, 1984). The technique is simple, rapid to perform, and capable of resolving DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation (Sambrook *et al.,* 1989)

Agarose, which is extracted from seaweed, is a linear polysaccharide (Sambrook *et al.,* 1989; Pharmacia, 1989). Agarose has large pores, is easy to prepare, and allows rapid run times. It can be poured into a variety of shapes, sizes and porosities, and can be run in a number of different configurations. The choices within these parameters depend primarily on the sizes of the DNA fragments to be separated. Although agarose gels have lower resolving power than polyacrylamide gels, they have a greater range of separation. DNAs of 200 bp to approximately 50 kb in length can be separated on agarose gels of various concentrations (Sambrook *et al.,* 1989). The location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide. Bands containing as little as 1-10 ng of DNA can be detected by direct examination of the gel in ultraviolet light (Sharp *et al.*, 1973).

Agarose gels are usually run in an horizontal configuration in an electric field of constant strength and direction. When an electric field is applied across the gel, DNA which is negatively charged at neutral pH migrates towards the anode. The rate of migration is determined by the following factors.

(a) Molecular size of the DNA

Molecules of linear double-stranded DNA, which tend to become oriented in an electric field in an end-on position (Fisher and Dingman, 1971; Aaij and Borst, 1972), migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs (Helling *et al.*, 1974). Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than the smaller molecules (Sambrook *et al.* , 1989).

(b) Agarose concentration

A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose (Sambrook *et al.*, 1989). There is a linear relationship between gel concentration and $\log_{10}$ of the electrophoretic mobility of DNA (Sambrook *et al.*, 1989; Pharmacia, 1989). Thus, by using gels of different concentrations (different porosities), it is possible to resolve a wide range of DNA molecules.

(c) Conformation of DNA

The different conformations of DNAs, superhelical circular (form I), nicked circular (form II), and linear (form III) of the same molecular weight, migrate through agarose gels at different rates (Thorne, 1967). The relative mobilities of the three forms depend primarily on the agarose concentration in the gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of the superhelical twists in the form I DNA (Johnson and Grossman, 1977).

(d) Applied voltage

Both the separation and resolution of DNA fragments are affected by the voltage gradient (Southern, 1979; Pharmacia, 1989). At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA increases differentially. Thus, the effective range of separation in agarose gels decreases as the voltage applied is increased (Sambrook *et al.*, 1989).

A balance has to be struck between resolution and separation. Low-molecular-weight fragments diffuse and are thus best separated at fairly high-voltage gradients. Large fragments, however, diffuse very slowly, and best resolution is achieved by using low-voltage gradients and running for long times (McDonell *et al.*, 1977; Phamacia, 1989).

(e) Direction of the electric field.

DNA molecules of larger than 50-100 kb in length migrate through agarose gels at the same rate if the direction of the electric field remains constant (Smith and Cantor, 1987; Sambrook *et al.*, 1989). However, because of the sieving effect of the gel matrix, if the direction of the electric field is altered periodically, the DNA molecules are forced to change course. The time it takes for a molecule to reorient itself in the new electric field depends on its length (Smith and Cantor, 1987). Because larger molecules take longer to reorient to the new direction of the field, pulse-field gel electrophoresis can be used to fractionate populations of extremely large molecules of DNA, up to about 10,000 kb (Smith and Cantor, 1987; Sambrook *et al.*, 1989).

(f) Base composition and temperature.

The electrophoretic behavior of DNA in agarose gels, in contrast to polyacrylamide gels (Allet *et al.*, 1973), is not significantly affected by either the base composition of the DNA (Thomas and Davis, 1975) or by the temperature at which the gel is run (Sambrook *et al.*, 1989). Most agarose gels are run at room temperature as the relative electrophoretic mobilities of DNA fragments of different size do not change between 4°C and 30°C. However, gels containing less than 0.5% agarose and low-melting temperature agarose gels are rather flimsy, and are best run at 4°C, where they are stronger (Sambrook *et al.,* 1989).

(g) Presence of intercalating dyes

Ethidium bromide, a fluorescent dye that is used to detect DNA in agarose and polyacrylamide gels, reduces the electrophoretic mobility of linear DNA by about 10-15% (Perbal, 1984; Sambrook *et al.,* 1989). The dye intercalates between stacked base pairs, extending the length of linear and nicked circular DNA and making them more rigid (Sambrook *et al.,* 1989).

(h) Composition of the electrophoresis buffer

The composition and ionic strength of electrophoresis buffers affect the mobility of DNA (Sambrook *et al.,* 1989). In the absence of ions, electrical conductance is minimal and the DNA migrates slowly, if at all. In buffers of high ionic strength, electrical conductance is very efficient and significant amounts of heat are generated. If overheating should occur, the DNA bands will be distorted, the DNA denatured or the gel melts (Sambrook *et al.,* 1989).

Several different buffers are available for electrophoresis of double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50 mM (pH 7.5-7.8) (Sambrook *et al.,* 1989).

(VI) DETERMINATION OF DNA FRAGMENT SIZES.

Estimation of molecular size of nucleic acids in acrylamide and agarose gel electrophoresis is important for the identification and the characterisation of restriction fragments and for studies of native and denatured DNA.

To estimate the size of DNA fragments from their mobilities in gel electrophoresis, a relationship is established between the mobilities and the lengths of standard fragments (Southern, 1979; Duggleby *et al.,* 1981; Elder and Southern, 1983). This relationship is then used to calculate the lengths of unknown fragments from their mobilities. The accuracy with which fragment lengths can be estimated depends on the accuracy of the chosen relationship between mobility and length (Duggleby *et al.*, 1981; Elder and Southern, 1983; Rochell *et al.*, 1985; Oerter *et al.*, 1990). Numerous methods have been proposed for graphical and computer analyses of the relationships between mobility and length (Duggleby *et al.*, 1981; Elder and Southern, 1983; Rochell *et al.*, 1985; Oerter *et al.*, 1990). The most commonly used is applied by plotting the logarithm of length against the mobility of standard fragments, and estimating the lengths of unknown fragments from the resulting graph (Duggleby *et al.*, 1981; Schaeffer and Sederoff, 1981; Elder and Southern, 1983). The standard curves obtained from such plots often show pronounced curvature which may introduce significant subjectivity into the interpolation (Duggleby *et al.*, 1981; Elder and Southern, 1983). Due to this, linear models have been devised that more or less fit with experimental data (Aaij and Borst, 1972; Duggleby *et al.*, 1981; Schaeffer and Sederoff 1981; Elder and Southern, 1983). However, none of these has been generally accepted, and most workers continue to use graphical or visual methods which are subjective and fail to provide estimates of the precision of the calibration curves or of the size estimate of the unknown fragments.

CHAPTER TWO

2.0 METHODOLOGY

2.1 THE ISOLATION, PURIFICATION AND MAINTENANCE OF YEAST CULTURES.

(I) RECOVERY OF YEAST FROM PALMWINE AND OTHER SOURCES

PROTOCOL

Palmwine samples were obtained from different locations in the southern parts of the country (see Fig. 1) and designated as shown in Table 1. Each sample was transferred into a labelled and sterilised centrifuge tube and centrifuged at 700 *g* for 10 minutes at 4°C. The supernatants were discarded and the pellets were washed twice with sterile tap water by means of centrifugation at 1600 *g* for 10 minutes. The supernatants were discarded after each wash. After the last wash, the tubes containing the pellets were stored at 4°C. The samples obtained from other sources namely pito (PT/B/1) and nmeda (N/B/1) were treated in like manner.

TABLE 1. DESIGNATION AND SOURCE OF ISOLATES.

DESIGNATION	SOURCE
PW/B/1	Akyem Sekyere
PW/B/2	Kibi
PW/B/3	Adeiso
PW/B/4	Aduagyiri
PW/B/5	Nsawam
PW/B/6	Frankadua
PW/B/7	Kumasi
PW/B/8	Tesano
PW/B/9	Adabraka
PW/B/10a	Legon
PW/B/10b	Legon
N/B/1	Osu
PT/B/1	Nima

Fig. 1. Map of Ghana showing the localities from which the palmwine samples were collected.

KEY

NUMBER	LOCATION
1	Akyem Sekyere
2	Kibi
3	Adeiso
4	Aduagyiri
5	Nsawam
6	Frankadua
7	Kumasi
8	Tesano
9	Adabraka
10	Legon

(II) PURIFICATION OF YEAST CULTURES.

Yeasts seldom occur in the absence of either moulds or bacteria. To obtain pure or axenic cultures of isolates, selective media, which, while permitting the development of yeasts, suppress mould and bacteria growth, are used. The composition of such media is determined by the fact that yeasts are, as a rule, capable of development at hydrogen-ion concentrations which do not favour bacteria growth.

PROTOCOL

The yeasts were isolated by direct plating of suspensions of the pellets unto YPD-agar plates (see Appendix A).

An inoculating loop was flamed and allowed to cool. The loop was then used to transfer a loopful of yeast pellet into a test-tube containing 5 ml of sterile distilled water and the suspension was thoroughly stirred. The even suspension was then employed as source material for streaking the YPD-agar plates. The sides of the plates were sealed with cellophane, the plates were inverted and incubated at room temperature (25-28°C) for 2-3 days. A small amount of yeast cells was also taken from the suspension, transferred to a microscopic slide and stained (see section 2.2, iii).

Colonies developing on these primary plates were inspected for their macromorphology under low magnification (x16). Selected, single, well isolated colonies were then brought into culture by replating. These were also incubated under the same conditions for 2-3 days. Cells taken from these colonies were also stained. After the incubation period, cells were taken from these secondary plates and stained.

(III) MAINTENANCE OF PURE CULTURES.

Axenic or pure cultures of isolates have to be properly maintained if they are to remain viable for any length of time. Maintenance of axenic cultures is also important in order that the same strains, rather than other isolates, are always used to ensure reproducible results.

Storage at 4°C prolongs survival of many organisms as a result of reduced metabolism. The majority of yeasts may be stored at 0-4°C and subcultured at 5-6 monthly intervals (van der Walt and Yarrow, 1987). Storage on agar slopes requires no laboratory equipment other than a refrigerator and is, moreover, convenient if inocula are frequently required.

PROTOCOL

The yeast isolates were maintained on MYPD-agar slopes (see Appendix B). A plate with axenic culture was examined and the colony to be transferred selected. An inoculating loop was flamed and allowed to cool. The cover of the plate was lifted, the colony touched with the loop and the cover replaced. The screw cap from the McCartney bottle was removed, the loop inserted and drawn along the surface of the slope from bottom to top. The loop was withdrawn and the screw cap was replaced. The slope was incubated at 25°C for 24 hours. The screws were tightened at the end of the incubation period and the bottle was stored at 4°C. For each isolate, two slopes were inoculated. One bottle was used as a source for routine inocula over a 4-6 month period; the other was preserved unopened to provide inoculum for the next pair of slopes for the following 4-6 months.

2.2. CLASSIFICATION AND IDENTIFICATION OF YEASTS

Newly isolated yeasts have to be identified to ensure that they are indeed the correct organisms and not contaminants.

Classification and identification of yeasts is based on a systematic study of their morphological, cultural, sexual and physiological characteristics which have been made under standard conditions.

(i) CHARACTERISTICS OF VEGETATIVE CELLS

(a) Morphological and cultural characteristics of vegetative cells grown on solid media.

PROTOCOL

Determination of the morphology and cultural characteristics of the vegetative cells on solid media were carried out on YPD-agar plates (see Appendix A).

Cells from actively growing slant cultures were used to inoculate the plates under aseptic conditions as under section 2.1, ii. After the streakings, the plates were inverted and incubated at 25°C for 3-5 days. The shape and mode of reproduction of the cells, as well as, the cultural characteristics of the growth were noted and recorded.

(b) Morphological and cultural characteristics of vegetative cells grown in liquid media

PROTOCOL

For these purposes YPD-broth media (see Appendix A) were used. Material from actively growing slants was inoculated in 10 ml of YPD-broth in 50 ml, cotton-plugged Erlenmeyer flasks. After 2-3 days incubation at 25°C the cultures were examined. The shape, mode of reproduction and cultural characteristics were noted. The cultural characteristics were noted again after 3 weeks incubation at the same temperature. The results of these observations were recorded.

(c) Cytological method

Within the visible spectrum of light with wavelengths between 400-700 nm, the human eye is able to detect variations in intensity and colour. Since yeast cells are essentially transparent to the visible spectrum of light and even structures such as vacuoles, nuclei and mitochondria show little contrast, a clear definition of cellular detail can only be obtained by increasing the contrast.

One way of increasing contrast is to use dyes that selectively stain the required structures or components of the cell. Stains are used to obtain more information of the shape, anatomy and taxonomic features of cells which are not easily seen in unstained materials. Staining has the advantage of providing permanent records of materials for repeated examination. Yeast cells can be stained for general observation by some of the stains used for bacteria.

PROTOCOL

Jensen's modification of Gram stain was used (Collins and Lyne, 1987). An inoculating loop which had been sterilised by flaming and cooling, was used to transfer a loopful of the sample on to a labelled, clean and grease-free slide. Using the flat of the loop, the transferred sample was smeared evenly over the central area of the slide. The slide was allowed to air-dry then heat-fixed by passing the slide over a flame from a spirit lamp. This caused the cells to adhere firmly to the slide. The slide was now covered with crystal violet solution (see Appendix C) for 20 seconds; the stain was washed off with water, followed by application of iodine solution (see Appendix C). The slide was next covered with fresh iodine solution for about 30 seconds. The iodine was washed off with absolute ethanol until colour ceased to come out of the preparation. The slide was then washed with water and then counterstained with neutral red solution (see Appendix C) for 1-2 minutes; it was washed with water and air-dried.

A drop of immersion oil was placed on the stained slide and the slide was examined carefully under the microscope using the oil immersion objective lens. Samples for examination of cellular morphology and mode of reproduction were taken from both YPD-agar and YPD-broth media for each yeast isolate. The results were noted and recorded.

(d) Formation of pseudomycelium and true mycelium.

PROTOCOL

The Dalmau plate technique was used with potato agar (potato-glucose agar) as the medium (see Appendix D).

Freshly poured plates were set aside for 1-2 days in order to allow their surfaces to dry. A single streak inoculation, was made near one side of the plate (as from the relative positions 10 o'clock to 12 o'clock). The inoculum was light and taken from a fresh slant culture. In addition to the single streak inoculation, two point inoculations were made near the other side of the plate (as at the positions 4 o'clock and 8 o'clock). A central section of the streak and one of the point inoculations were covered with sterile coverslips. The preparations were studied microscopically using a 3-mm dry objective and x10 oculars after incubation at 25°C for 7-10 days.

(ii) SEXUAL CHARACTERISTICS

Characteristics of ascospore formation

PROTOCOL

The culture to be studied was first brought into a state of active growth and optimal nutrition by subculturing on a special pre-sporulation medium (see Appendix E) for two days at 25-28°C. The sporulation medium (see Appendix E) was then lightly inoculated with the culture.

The inoculated sporulation media were then incubated at 25°C for 3 days before being examined microscopically for the first time. Material that had not sporulated was then maintained at room temperature and examined after a week. Ascospore formation was verified by staining heat-fixed preparations using Schaeffer-Fulton's modification of the Wirtz method (Schaeffer and Fulton, 1933).

Heat-fixed preparations were flooded with 5% aqueous malachite green (see Appendix E) for 30-60 seconds, and heated to steaming 3-4 times. The excess stain was rinsed off under running tap water for about half a minute. The preparations were then counterstained with 0.5% safranine (see Appendix E) for about 30 seconds. The stained preparations were examined microscopically.

(iii) PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

(a) Fermentation of carbohydrates

PROTOCOL

The strains to be tested were first brought into a state of active growth. This was effected by transferring the strains once on YPD-agar (see Appendix A) at 25-28°C for 2-3 days.

2.0 ml aliquots of the fermentation basal medium (see Appendix F) were pipetted into sterile, plugged 150 x 12 mm tubes carrying insert tubes of approximately 50 x 6 mm which had been sterilised by autoclaving (15 minutes at 121°C). After the sterilisation, 1 ml quantities of 6% (v/v) sterile solutions of the requisite sugars (glucose, galactose, sucrose, maltose and lactose), were aseptically transferred into the tubes.

The tubes were inoculated directly from the actively growing cultures by means of a stout platinum loop. The tubes were incubated at 25°C, regularly shaken and observed for the accumulation of gas in the insert tubes and for a change in colour of the indicator used over a period of 5 days. Two blank tubes, one containing the 2 ml of basal medium plus 1 ml glucose solution and the other containing 2 ml of basal medium plus 1 ml of sterile distilled water, were also set up. The observations made were recorded

(b) Assimilation of nitrogen compounds

PROTOCOL

Aliquots of 18-20 ml of sterile, synthetic basal medium (see Appendix F) devoid of a nitrogen source, and which had been cooled to about 40°C, were poured into sterile Petri dishes containing about 2 ml of a suspension of the yeast under test in sterile tap water. The liquids were thoroughly mixed and the plates were allowed to set. After solidification, the plates were kept, lid-side up, at 25°C for a few hours to obtain dry agar surfaces. Small amounts of the various nitrogen compounds (potassium nitrate, tri-ethylamine hydrochloride, L-lysine and ammonium sulphate, see also Appendix F), were deposited at different, evenly spaced sites on the agar. These sites were marked on the outside of the smaller dish. The plates were then incubated at 25°C lid-side down. The results were observed after 3 days and recorded.

(c) Growth at 37°C

PROTOCOL

The cells to be tested were brought into a state of active growth by subculturing on YPD-agar plates (see Appendix A) at 25-28°C for 2-3 days.

The actively growing cultures were then lightly inoculated as streaks on MYPD-agar plates (see Appendix B) and incubated at 37°C for 2-4 days. The results of the observations made were recorded.

2.3 MOLECULAR GENETIC ANALYSIS

(i) YEAST CELL GROWTH

Most DNA experiments are performed with yeast cultures in the log phase of cell growth. Therefore, the concentration of yeast cells per volume and the approximate rate of growth must be known. Two basic methods for the determination of the concentration of microorganisms in a culture, namely, turbidimetric measurements and cell counting, were used.

(a) Turbidimetric measurements

The turbidity of a culture is a function of growth since it reflects increases in cell mass per unit volume of culture. The turbidity is due to light scattering, and is best measured at wavelengths where the ratio of absorbance to light scattering is low (Rodriguez and Tait, 1983). Changes in cell mass can be followed by measuring the turbidity of the culture with a colorimeter or a spectrophotometer.

For enumeration purposes, the results from turbidity measurements may be correlated with changes in cell numbers.

PROTOCOL

The yeast cells were grown in YPD-broth medium (see Appendix A). Material from actively growing slants were inoculated in 4 ml of sterile YPD-broth medium in 10 ml capped tubes under aseptic conditions. These were placed on a rotary shaker (Thermolyne Type 65800 Maxi Mix III) at 400-600 rpm overnight at room temperature (25-28°C); and served as starter cultures.

These starter cultures were used to inoculate (under aseptic conditions) 40 ml of the same medium in 100-ml conical flasks and the mouths of the flasks were loosely plugged with non-absorbent cotton-wool. The flasks were swirled round gently to evenly disperse the cells and placed on the rotary shaker at 200-300 rpm at room temperature. Using a sterile pipette tip, 200 μl of the cell suspension was taken from each flask and transferred into 1.8 ml of formaldehyde-saline solution (see Appendix G) to arrest cell growth. These mixtures were briefly vortexed and their absorbances read, using a

Shimadzu UV 190 Double Beam Spectrophotometer at 600 nm against a blank (200 µl YPD-broth + 1.8 ml of formaldehyde-saline solution) whose result was taken as absorbance reading for time zero. The same volumes of cell suspensions were taken at hourly intervals and treated in the same manner. The experiment was continued till the cell growth reached the log phase as determined on the graph of absorbance versus time (Fig. 4A) drawn during the progress of the experiment.

The cells were harvested at this phase by centrifugation, washed once by means of centrifugation in sterile 1.2 M sorbitol, and stored at -20°C.

(b) Cell counting (direct microscopic examination

The turbidity of a culture can only be used as an estimate of cell concentration after turbidity has been calibrated against a direct count of cells.

The number of cells in a given sample may be determined by microscopic examination of a portion of the material by counting the number of cells observed. By using an aliquot of known volume, the number of cells in the original sample can be calculated. The precision and accuracy of this method are related to the number of fields counted.

PROTOCOL

The cells were counted by the technique of haemocytometry recommended by the WHO (1988) using an Improved Neubauer Counting Chamber (Weber Scientific International, Lancing, England).

The haemocytometer was cleaned and dried. To ensure that the correct volume was attained, the cover-glass was firmly placed on top of the chamber so as to produce 3-5 Newton's rings. The counting chamber was filled, using a micropipettor, with an evenly dispersed suspension of cells in formaldehyde-saline solution taken at hourly intervals for the turbidimetric measurements. Care was taken to ensure that the counting chamber was completely filled in one action and that no fluid flowed into the surrounding moat. Counting was delayed for 2-3 minutes to allow the cells to settle. Both counting chambers were filled.

The haemocytometer was carefully transferred to the microscope stage and the preparation examined with a x10 eyepiece. Counting was done in five squares (see Appendix H) for both chambers. All cells lying on or touching two of the four sides (right and lower) were counted, while those on the other two sides (left and upper) were left uncounted. The concentration of cells in the original sample was obtained by calculation and a statistical evaluation obtained (see Appendix H). A graph of cell-number versus time was constructed from the data so obtained.

By combining the plot of turbidity versus time and the plot of cell count versus time, a standard curve (Fig. 4C) was constructed relating the two parameters, thus, giving a rapid means for measuring cell concentration.

(c) Yeast sphaeroplasting

The greatest difficulty in the isolation of highly polymerized DNA from yeast has been to obtain a relatively simple and gentle method of lysis of the yeast cell wall. Yeast cells can be lysed by mechanical disruption, or by freeze-thawing in sodium dodecyl sulphate (SDS), or by the formation of sphaeroplasts following enzymatic degradation of their cell walls and subsequent lysis in a detergent (Smith and Halvorson, 1967).

The last method is currently the method of choice since it is relatively simple and rapid, and unlike the other methods, can easily be monitored. Several different enzymes are commercially available, which can be used to digest the cell wall to produce intact sphaeroplasts, provided an osmotic stabilizer is present to prevent lysis.

PROTOCOL

The method used was as given by the manufacturers (Sigma Chemical Company, USA) of the commercial enzyme Lyticase (Sigma, L8137), and it was as follows. Yeast cells grown to the mid-log phase (2-5 x 10^7 cells/ml), in 40 ml of YPD-broth medium on a shaker at 200-300 rpm and at 25-28°C, were harvested by centrifugation (900 *g* for 5 minutes). An appropriate quantity of cells was resuspended in 1.2 M sterile sorbitol (see Appendix I) to give a A_{800} of approximately 6.0. One part of enzyme solution (see

Appendix I) was added to four parts of yeast cell suspension in a 5-ml polypropylene tube. A control, for the purpose of monitoring sphaeroplast formation, was prepared by adding one part of potassium phosphate buffer to four parts of yeast cell suspension. The suspensions were then incubated with shaking at 30°C in a water-bath (Eyela water-bath SB-24 with Eyela thermistor Tempett T-80, and Eyela shaker SS-8).

Sphaeroplast formation was monitored and confirmed by removing an aliquot from the enzyme exposed suspension, diluting it 10-fold in 10% SDS solution, and microscopically examining the suspension for the presence of cells. The absence of cells, indicating the completion of sphaeroplast formation, was normally observed after 45 minutes. The control suspension when treated as above, retained fully intact cells. The sphaeroplasts were centrifuged (2000 *g* for 5 minutes at 4°C) and washed once by means of centrifugation in 1.2 M sterile sorbitol.

(II) ISOLATION, PURIFICATION, AND QUALITY CONTROL OF YEAST GENOMIC DNA FOR RESTRICTION ANALYSIS.

For the purposes of getting readable and reproducible electrophoretic patterns after cleavage by restriction endonucleases, isolated DNA must be of high molecular weight and free from inhibitors that might interfere with endonuclease action. Isolation of DNA of high purity for restriction analysis is, therefore, a key step.

Since most DNA *in vivo* is present in association with RNA and proteins, it is necessary, first to isolate crude complexes from cells and then to purify and separate the DNA from the proteins and RNA. Purification of isolated DNA is most often easily achieved by cycles of phenol and chloroform extractions, RNase treatment, and ethanol precipitation. The purity of the DNA isolated can easily be verified through spectrophotometric analysis.

(a) Isolation and purification of genomic DNA

PROTOCOL

The following protocol was adapted from Struhl *et al*.. (1979), and Rodriguez and Tait (1983).

The sphaeroplasts were resuspended in 1.5 ml of 50 mM EDTA (pH 8.5, see Appendix J), followed by the addition of 60 µl of 10% SDS. The suspension was then heated for 15 minutes at 70°C in a water-bath and cooled to room temperature. An equal volume of phenol/ chloroform/ isoamylalcohol (25: 24: 1,v/v/v, see Appendix J) was added and mixed gently until the two solutions were homogeneous. The two phases were separated by centrifugation (13,000 *g* for 10 minutes at 4°C). Using a wide-bore pipette, the aqueous phase was transferred to a clean 5-ml centrifuge tube. An equal volume of chloroform/ isoamylalcohol (see Appendix J) was added to the aqueous phase and the process repeated as for the phenol/ chloroform/ isoamylalcohol step.

The aqueous phase obtained after this second extraction was transferred to a fresh tube and 2 volumes of cold absolute ethanol (-20°C) was added with gentle mixing. The solution was left on ice for 5 minutes with occasional swirling, followed by centrifugation (13,000 *g* for 10 minutes at 4°C). The supernatant was discarded, excess liquid was drained off and finally the DNA was air-dried for a few minutes. The DNA was dissolved in 1.5 ml of TE buffer (1mM EDTA, 10 mM Tris-HCl, pH 7.4, see Appendix J); 7.5 µl of RNase solution (10 mg/ml, see Appendix J) was added and incubated at 37°C for 1 hour. The DNA was precipitated by addition of 2 volumes of cold absolute ethanol (-20°C) to the aqueous phase and the tube left on ice for 1-2 hours. The DNA was pelleted by centrifugation (13,000 *g* for 10 minutes at 4°C), the supernatant was discarded, excess liquid was drained off and the pellet air-dried. The pellet was resuspended in 100 µl of TE buffer (1mM EDTA, 20 mM Tris-HCl, pH 7.4 see Appendix J) and kept at 4°C.

(b) Quantitation and purity of DNA

Maximum absorption of DNA is at 260 nm, whereas that of protein is at 280 nm. At 260 nm, proteins still absorb ultraviolet light . However, the contribution of proteins to absorbance at 260 nm is roughly equivalent to that measured at 300 nm (Grimont and Grimont, 1991).

For quantitating the amount of DNA, readings are taken at wavelengths of 260, 280 and 300 nm. The absorbance ratio A_{260}/A_{280} provides an estimate of the purity of the nucleic acid. The ratio should be about 1.8-2.0. Ratios less than these values indicate significant contamination with protein. If the ratio of A_{260}/A_{280} is 1.8-2.0 and the A_{300} close to zero, an A_{260} of 0.2 corresponds to 10 μg DNA/ml.

PROTOCOL

The method was adapted from Rodriguez and Tait (1983). 10 μl of DNA sample was diluted with 1.9 ml of distilled water and the absorbances at 260, 280 and 300 nm read in silica cuvettes against a distilled water blank on a double-beam spectrophotometer (Shimadzu UV 190 Double Beam). From the absorbance readings obtained, the quantity and purity of the DNA samples were calculated.

(III) CLEAVAGE OF DNA WITH RESTRICTION ENDONUCLEASES

PROTOCOL

The method was adapted from Sambrook *et al.* (1989), from Grimont *et al.* (1991) and from the manufacturer's instructions.

The DNA solutions were placed in sterile microfuge tubes and mixed with sufficient sterile distilled water to give a volume of 18 μl and a concentration of 2-5 μg DNA. To these were added 2 μl of the appropriate 10x restriction digestion buffers (see Appendix K) and the contents of the tubes were mixed by tapping. When all the tubes were ready, the appropriate enzyme (see Appendix K) was removed from the freezer, put immediately on ice, and 1 μl dispensed in the appropriate tube by pipetting directly into the contents of the reaction tubes. A fresh, sterile pipette tip was used every time an enzyme was dispensed, keeping the enzyme on ice whilst doing so. The enzymes were returned to the freezer immediately after use. The reaction tubes were tapped to mix their contents and spun for a few minutes to collect all liquid at the bottom of the tubes. All the tubes were incubated at 37°C in a water-bath (Eyela SB-24 with Eyela thermistor Tempett T-80) overnight .

After the period of incubation, the tubes were spun for a few seconds to collect evaporated water that had condensed on the tube caps. To each tube, 5 µl of gel loading solution (see Appendix K) was added to stop the enzyme reaction, the contents were well mixed and the tubes were spun for a few seconds to remove air bubbles, before loading the samples on a gel. Reaction mixtures which could not be loaded on a gel on the same day were stored at -20°C .

(IV) AGAROSE GEL ELECTROPHORESIS OF DNA RESTRICTION FRAGMENTS

Electrophoresis through agarose gels is a standard method used to separate and identify DNA fragments. The technique is simple, rapid to perform and capable of giving very fine resolution of fragments. The location of DNA within the gel is determined by staining with a low concentration of ethidium bromide and by examination of the gel in ultraviolet light .

Many designs of agarose gel electrophoresis equipment have been described (Southern, 1979). Horizontal agarose gels have won favour over vertical gels for the following reasons: gels are very simple to load, pour and handle ; they can easily be cast in a variety of thicknesses and at low concentrations (because they are supported from below).

DNA molecular weight markers are run along with the DNA restriction fragments to aid interpolate the sizes of the unknown DNA fragments.

PROTOCOL

The method was adapted from Sambrook *et al* (1989) and manufacturer's instructions. The electrophoresis chamber was placed in proximity to the power supply (Biorad Model 200/2.0) with which it was to be connected. Using the built-in bulls eye level and the adjustable feet, the chamber was levelled at the location where the gel was to be run. Both ends of the gel tray (10 x 6.5 x 0.5 cm) were sealed with adhesive tape and the tray was placed across the chamber. The depth gauge surface to be used was selected and the well-forming comb carefully positioned to ensure that the comb was level and also in the correct position for gel casting.

Sufficient electrophoresis buffer (0.5x TBE, see Appendix L) to fill the electrophoresis chamber and to prepare the gel was made up. The agarose was weighed and added to the correct volume of buffer to prepare a 0.7% gel in a conical flask. The slurry was heated in a boiling water bath, with swirling from time to time, until the agarose was completely dissolved. The solution was cooled to 50°C and ethidium bromide (see Appendix L) was added to give a final concentration of 0.5 μg/ml, and the mixture thoroughly mixed. The molten agarose solution was poured into the gel tray to a depth of approximately 0.3-0.5 cm. Using a plastic probe, any trapped air bubbles in the gel solution were removed and the gel was allowed to completely cool and solidify. After the gel was completely set, both adhesive tapes and the comb were carefully removed and the gel was positioned in the chamber with the sample wells oriented closest to the negative electrode. The chamber was slowly filled with just enough electrophoresis buffer solution to cover the gel to a depth of about 1 mm.

The DNA samples, mixed with gel-loading buffer, were carefully loaded into the slots of the submerged gel using a micropipettor. The two outermost slots were loaded with DNA molecular weight markers (see Appendix L). The safety cover was placed onto the chamber with both banana plugs securely attached; the attached leads were connected to the electrophoresis power supply so that the DNAs migrated toward the anode. A voltage of 100 V was applied and the gel run until the bromophenol blue tracking dye had migrated to the other end. Thereafter, the current was turned off and the safety cover removed from the chamber. The gel was placed on the UV transilluminator (Ultra-lum UVA 40 Dual Intensity), with the blocking shield in place, and examined.

(V) PHOTOGRAPHING THE GELS

Photographs of the gels were made using the transmitted light from the UV transilluminator.

The camera (Model QSP Instant Camera), with hand grip and orange filter attached, was loaded with the film (Polaroid Type 667) according to the manufacturer's instructions. The hood (Model QSP Hood No. 14) was now attached and the whole set-up placed completely over the gel (with UV luminescent ruler by the side), allowing it to rest completely on the UV table. The appropriate shutter speed and lens aperture were set, the light source was turned on and the trigger squeezed. The film was processed as recommended by the manufacturers. The results are shown in Fig. 5 and Figs. 6A-6J.

(VI) DETERMINATION OF DNA FRAGMENT SIZES

The method was adapted from Duggleby *et al* (1981). This is a computer programme that has been developed for determining the sizes of DNA restriction fragments from their electrophoretic mobilities. The programme fits a parabola to a set of standard fragments of known size and prints information on the adequacy of the fitted curve as the actual and calculated mobility of the standards, as well as their true and estimated sizes and the standard error of the fit. Using this fitted curve the programme calculates the size of an unknown fragment from its mobility.

PROTOCOL.

The programme, which had been written in BASIC and developed under the MULTI-USER BASIC/ TR-11 operating system on a PDP 11/ 34A, was modified and run on an Apple IIGS (model ROM 01) and written in Applesoft BASIC.

Two sets of size standards (see Appendix L) were used. The measurements of mobilities were taken from photographic enlargements of the gel photographs taken under section vii. above. Migration distances were measured from the visible origin of the gel (the gel slots) directly on the photographs. The mobilities were converted to molecular weights by the computer programme. This was done for each restriction enzyme digest for all the isolates. The results are shown in Table 8 (see Appendix N).

Regression analysis was used to obtain an estimate of the standard error of fit. The computer programme used is given in Appendix M.

CHAPTER THREE

3.0 RESULTS

3.1 ISOLATION, PURIFICATION AND MAINTENANCE OF YEAST CULTURES.

Microscopic examination of the fresh palmwine samples showed many yeast cells and rod-shaped bacteria (Fig. 2). This flora was constant for all the samples obtained. As the yeasts were bottom-fermenters, centrifugation at the low speed of 700 *g* for 10 minutes was enough to recover a high number of yeast cells from the palmwine.

The yeasts were isolated by direct plating as they were in high numbers. The development of moulds was restricted by the exclusion of air by sealing edges of the plates with cellophane. This favoured the development of the fermentative palmwine yeasts. Two platings were adequate to obtain axenic cultures. With the exception of the sample from Legon from which two isolates were obtained, only single isolates were obtained from the other nine isolates studied.

No loss of activity was observed on storage of the axenic cultures on MYPD-agar slopes at 4°C after a period of 5-6 months.

3.2 CLASSIFICATION AND IDENTIFICATION OF YEASTS.

(i) MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF VEGETATIVE CELLS GROWN ON SOLID AND IN LIQUID MEDIA.

All the yeast cells showed positive staining with Gram's stain. The shape of the cells, globose, ellipsoidal, or cylindrical was the same, on both solid and in liquid media, for isolates PW/B/1, PW/B/2 through to PW/B/9 (Figs. 3A), PW/B/10a and also for PT/B/1 (Fig. 3D). Isolate PW/B/10b had a longitudinal cell shape (Fig.3C), whilst the nmeda isolate N/B/1 had an apiculate cell shape (Fig.3E). The mode of vegetative reproduction was by multilateral budding for all the isolates, except N/B/1, whose reproduction was by bipolar budding.

All the isolates, PW/B/1,PW/B/2 through to PT/B/1, showed consistent cultural characteristics on solid and in liquid media. The cultural characteristics exhibited by their colonies (Table 2), especially, the semi-glossy surfaces, are special morphological characteristics of *Saccharomyces* species (MacMillan and Phaff, 1978). In liquid media, the presence of a sediment, a ring, and the absence of a pellicle after 3-4 weeks (Table 3) are also characteristics of *Saccharomyces* species. Isolate N/B/1 showed characteristics different from those observed for the others (Tables 2 and 3).

(II) ASEXUAL AND SEXUAL CHARACTERISTICS.

None of the isolates formed mycelia on potato-dextrose agar. However, PW/B/3, PW/B/4, PW/B/5, PW/B/6 and PW/B/8 formed pseudomycelia (Table 4).

Ascospores were formed by all the isolates, with exception of N/B/1. The shape and number of ascospores formed by PW/B/1, PW/B/2 through to PW/B/8 were the same (Table 4). PT/B/1 formed ascospores which were bigger, rounder and fewer in number per ascus (Table 4). In all cases, the ascospores were not liberated and the asci were also unconjugated .

(III) PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS.

(a) Fermentation of carbohydrates

Fermentation tests showed that all the isolates fermented glucose vigorously, but none fermented lactose (Table 5).

Isolate N/B/1 did not ferment any other sugar apart from glucose. On the other hand, the others showed an equal vigorous fermentation of sucrose and maltose. The only difference observed in the fermentative abilities of these isolates was in their fermentation of galactose (Table 5). For PW/B/1, PW/B/2, PW/B/7 and PT/B/1, there was no gas production although there was a change in the colour of the indicator used. For PW/B/3, PW/B/4, PW/B/5, PW/B/6, PW/B/8 and PW/B/9, there was both gas production and a change in the colour of the indicator used.

(b) Assimilation of nitrogen compounds.and growth at 37 °C

All the isolates were unable to utilize nitrate-nitrogen. They were all also unable to utilize both ethylamine hydrochloride and L-lysine as sole sources of nitrogen (Table 6), while ammonium sulphate which was used as a control was utilized.

All the isolates also grew vigorously at a temperature of 37°C.

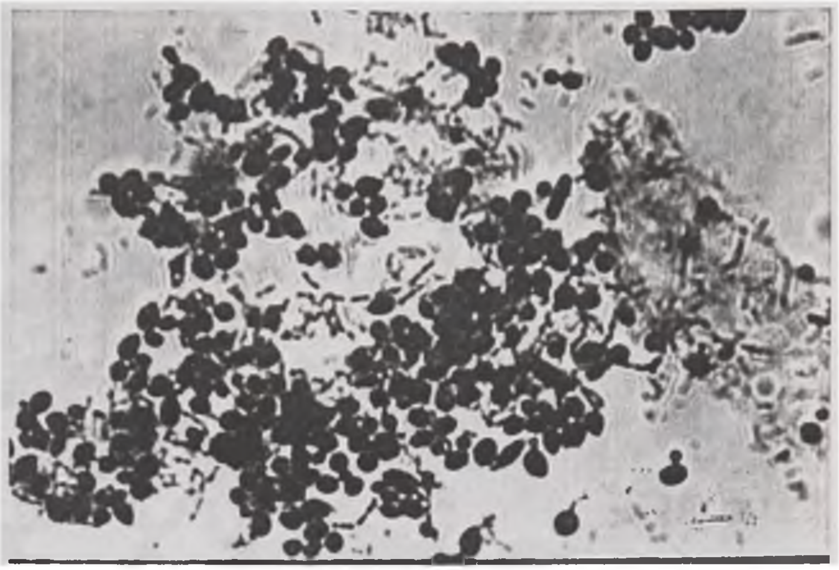


FIG. 2. Yeast cells from fresh untreated palmwine samples. Rod-shaped bacteria cells can also be seen (Mag. x100)

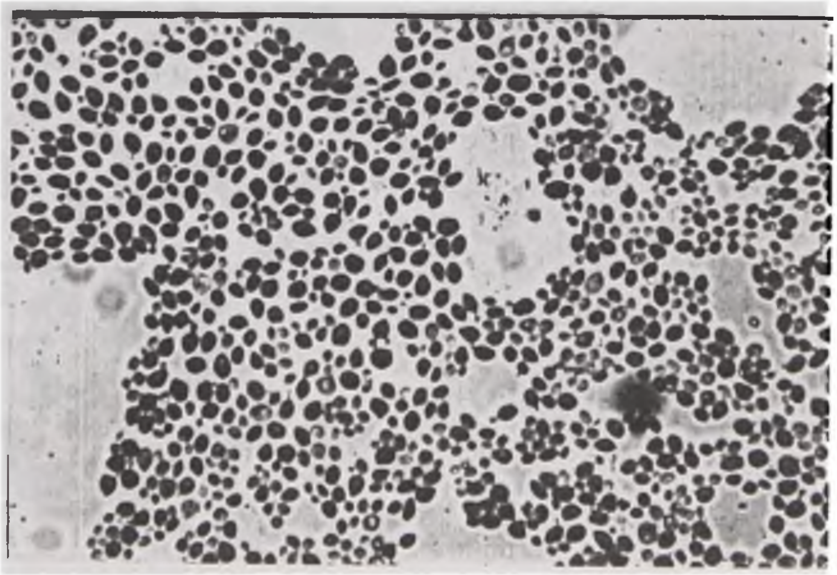


FIG. 3A. Isolates PW/B/1 , PW/B/2 through to PW/B/9. Yeast cells obtained from palmwine samples (Mag. x100)

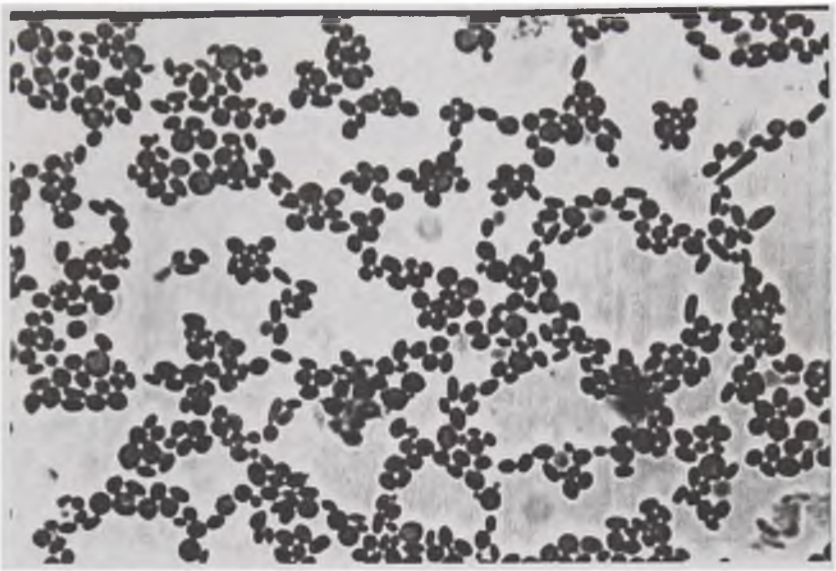


FIG. 3B. Isolate PW/B/10a. Yeast cells from palmwine sample from Legon. (Mag. x100)

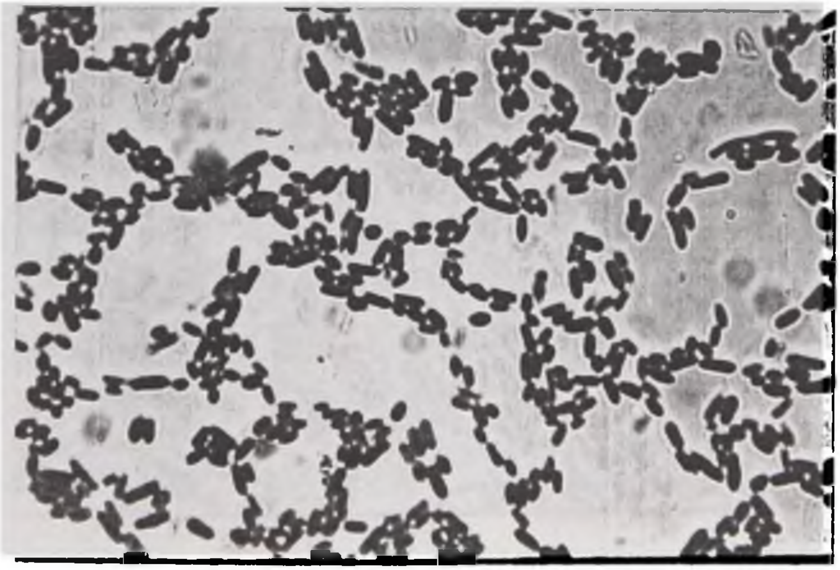


FIG. 3C. Isolate PW/B/10b. Yeast cells of different cell shape from palmwine sample from Legon.(Mag. x100).

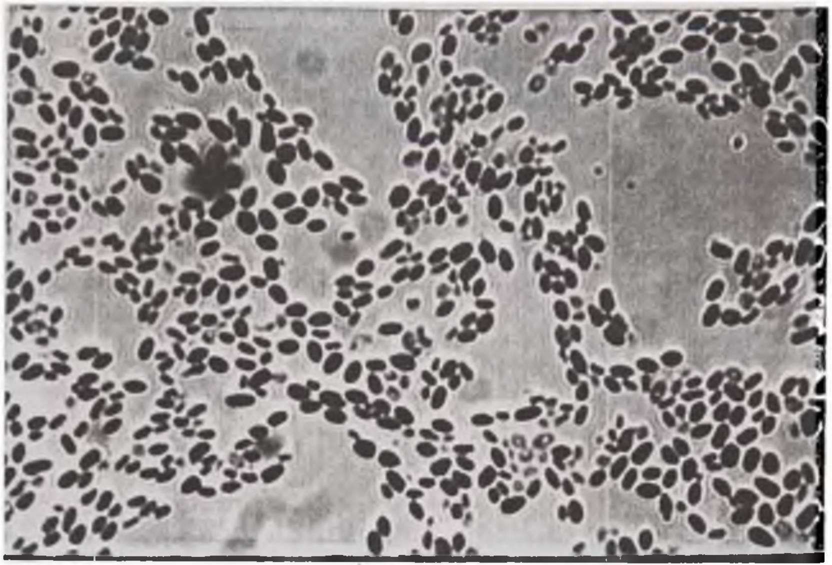


FIG. 3D. Isolate PT/B/10. Yeast cells from pito sample. (Mag. x100).

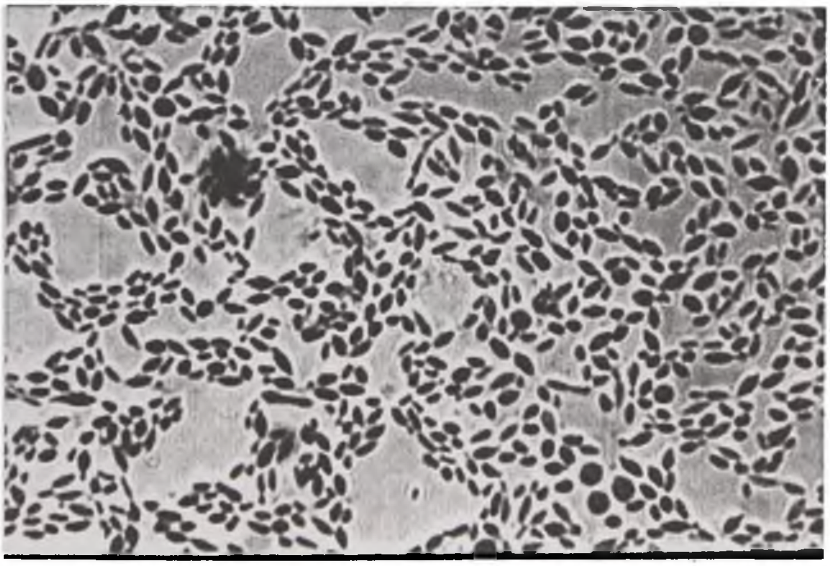


FIG. 3E. Isolate N/B/1. Yeast cells from nmeda sample. (Mag. x100)

TABLE 2: MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF YEAST ISOLATES ON YPD-agar.

ISOLATES	TEXTURE[a]	COLOUR[b]	SURFACE[c]	ELEVATION[d]	MARGIN[e]	CELL SHAPE[f]
PW/B/1	+	+	+	+	+	+
PW/B/2	+	+	+	+	+	+
PW/B/3	+	+	+	+	+	+
PW/B/4	+	+	+	+	+	+
PW/B/5	+	+	+	+	+	+
PW/B/6	+	+	+	+	+	+
PW/B/7	+	+	+	+	+	+
PW/B/8	+	+	+	+	+	+
PW/B/9	+	+	+	+	+	+
PW/B/10a	+	+	+	+	+	-
PW/B/10b	+	+	+	+	+	+
N/B/1	+	-	+	+	+	-
PT/B/1	+	+	+	+	+	+

a^+ = pasty: b^+ = white and creamy: c^+ = smooth and semi-glossyd^+ = raised and restricted: e^+ = smooth and entire: f^+ = spheroidal and globose, ellipsoidal: f^- = longitudinal

TABLE 3. MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF YEAST ISOLATES IN YPD-broth.

ISOLATES	SEDIMENT[a]	PELLICLE[a]	BUDDING[b]	CELL SHAPE[c]	RING[a]
PW/B/1	+	-	ML	A,B	+
PW/B/2	+	-	ML	A,B	+
PW/B/3	+	-	ML	A,B	+
PW/B/4	+	-	ML	A,B	+
PW/B/5	+	-	ML	A,B	+
PW/B/6	+	-	ML	A,B	+
PW/B/7	+	-	ML	A,B	+
PW/B/8	+	-	ML	A,B	+
PW/B/9	+	-	ML	A,B	+
PW/B/10a	+	-	ML	A,B	+
PW/B/10b	+	-	ML	C	+
N/B/1	+	-	BP	D	+
PT/B/1	+	-	ML	A	+

a^+ = present ; a^- = absent ;b^{ML} = multilateral budding; b^{BP} = bipolar budding c^A = spheroidal/ globose; c^B = prolate-ellipsoidal; c^C = longitudinal; c^D = apiculate

TABLE 4.ASEXUAL AND SEXUAL CHARACTERISTICS OF YEAST ISOLATES.

	ASEXUAL		SEXUAL	
ISOLATES	MYCELIUM^a	PSEUDO-MYCELIUM^a	ASCOSPORE SHAPE^b	ASCOSPORE NUMBER
PW/B/1	-	-	A	1-4
PW/B/2	-	-	A	1-4
PW/B/3	-	+	A	1-4
PW/B/4	-	+	A	1-4
PW/B/5	-	+	A	1-4
PW/B/6	-	+	A	1-4
PW/B/7	-	-	A	1-4
PW/B/8	-	+	A	1-4
N/B/1	-	-	A	1-2
PT/B/1	-	-	-	-

a^+ = present ; a^- = absent
b^A = spheroidal/ globose

TABLE 5. PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF YEAST ISOLATES: FERMENTATION OF CARBOHYDRATES.

ISOLATES	GLUCOSE^a	GALACTOSE^a	SUCROSE^a	MALTOSE^a	LACTOSE^a
PW/B/1	+	A	+	+	-
PW/B/2	+	A	+	+	-
PW/B/3	+	+	+	+	-
PW/B/4	+	+	+	+	-
PW/B/5	+	+	+	+	-
PW/B/6	+	+	+	+	-
PW/B/7	+	A	+	+	-
PW/B/8	+	+	+	+	-
PW/B/9	+	+	+	+	-
N/B/1	+	-	-	-	-
PT/B/1	+	+	+	+	-

a^+ = strong fermentation, gas produced, colour change
a^A = fermentation, no gas produced, colour change
a^- = no fermentation, no colour change

TABLE 6: PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF YEAST ISOLATES: ASSIMILATION OF NITROGEN COMPOUNDS AND GROWTH AT 37 °C.

	ASSIMILATION OF NITROGEN COMPOUNDS				
ISOLATES	$(NH_4)_2SO_4$	KNO_3	Ety-HCl	L-Lysine	GROWTH AT (37^{0}C)
PW/B/1	+	-	-	-	+
PW/B/2	+	-	-	-	+
PW/B/3	+	-	-	-	+
PW/B/4	+	-	-	-	+
PW/B/5	+	-	-	-	+
PW/B/6	+	-	-	-	+
PW/B/7	+	-	-	-	+
PW/B/8	+	-	-	-	+
N/B/1	+	-	-	-	+
PT/B/1	+	-	-	-	+

3.3 MOLECULAR GENETIC ANALYSIS

(I) YEAST CELL GROWTH AND SPHAEROPLASTING.

Preliminary studies showed that the yeast isolates had a relatively higher absorbance at 660 nm than at 600 nm (which is normally used); consequently, all turbidity measurements were taken at 660 nm. These studies also showed that to obtain a high yield of cells within 24 hours in a 40 ml culture, an overnight start culture of 4-6 ml was required.

With the exception of isolate PW/B/10b, all the isolates showed the same growth curves of absorbance versus time of incubation (Fig. 4A) and cell density (concentration) versus time (Fig. 4B). From the two plots, the standard curve (Fig. 4C) was constructed relating absorbance at 660 nm against cell concentration, thus providing a rapid means for estimating cell concentration.

Following the manufacturers instructions, sphaeroplasts were obtained between 45-60 minutes after incubation at 30°C. It was observed that a slow shaking of the reaction flask enhanced sphaeroplast formation .

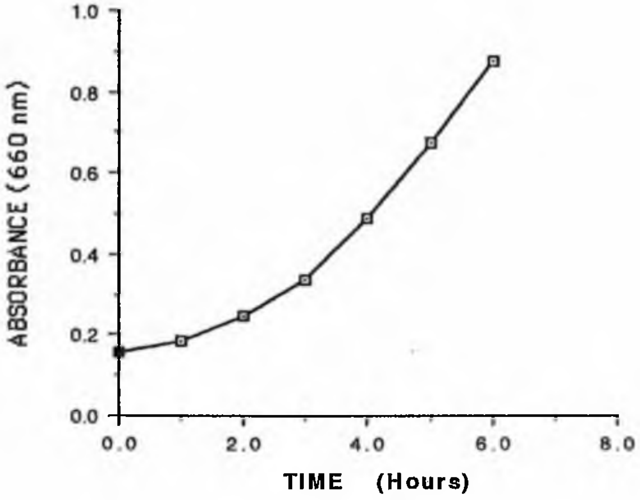


Fig. 4A. Yeast cell growth plotted as a function of absorbance at 660 nm versus time of incubation.

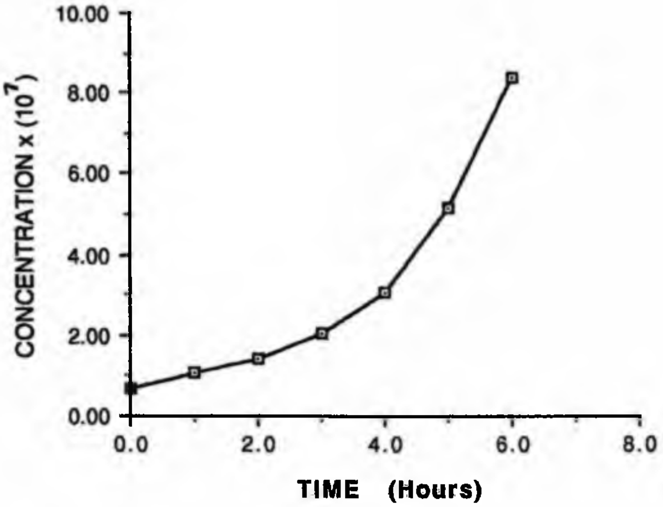


Fig. 4B. Yeast cell growth plotted as a function of cell concentration versus time of incubation.

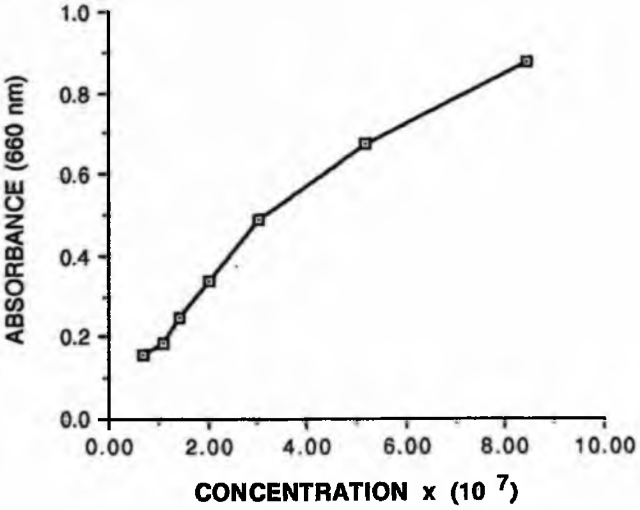


Fig. 4C Standard curve relating absorbance to cell concentration.

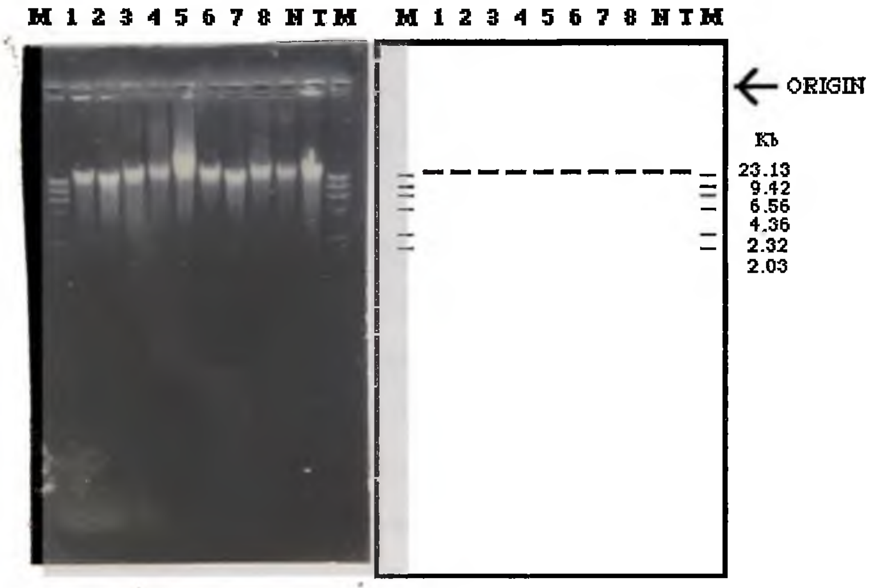
(II) ISOLATION AND PURIFICATION OF GENOMIC DNA.

The single most critical component of a restriction endonuclease reaction is the degree of purity of the DNA substrate (Fuchs and Blakesley, 1983). Improperly prepared DNA samples will be cleaved poorly, if at all, producing partially digested DNA.

DNA isolated by the protocols used was found to be of adequate purity for restriction analysis. The yield of DNA isolated was in the range of 4.4-10.2 µg/ml from a 40 ml culture ($2\text{-}5 \times 10^{7}$ cells/ml).

Analysis for purity using UV absorption spectrophotometry gave A_{260}/A_{280} ratios of between 1.4-1.6 and also an A_{300} of between 0.032-0.045.

In standard 0.7% agarose gel electrophoresis, the DNA isolated, when uncleaved, migrated as one band slightly slower than the 23.1 bp marker fragment of λ DNA cleaved with *Hind* III (Fig. 5). Under these conditions DNA from 25 to over 500 kb migrates as one band (Philippsen *et al.,* 1991).



a. b.

Fig. 5.(a) Undigested DNA from the yeast isolates. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1 **(b).** A schematic representation of the gel shown in **a.** (Lines in bold represent strong bands).

(III) RESTRICTION ENZYME DIGESTION PATTERNS OF TOTAL GENOMIC DNA.

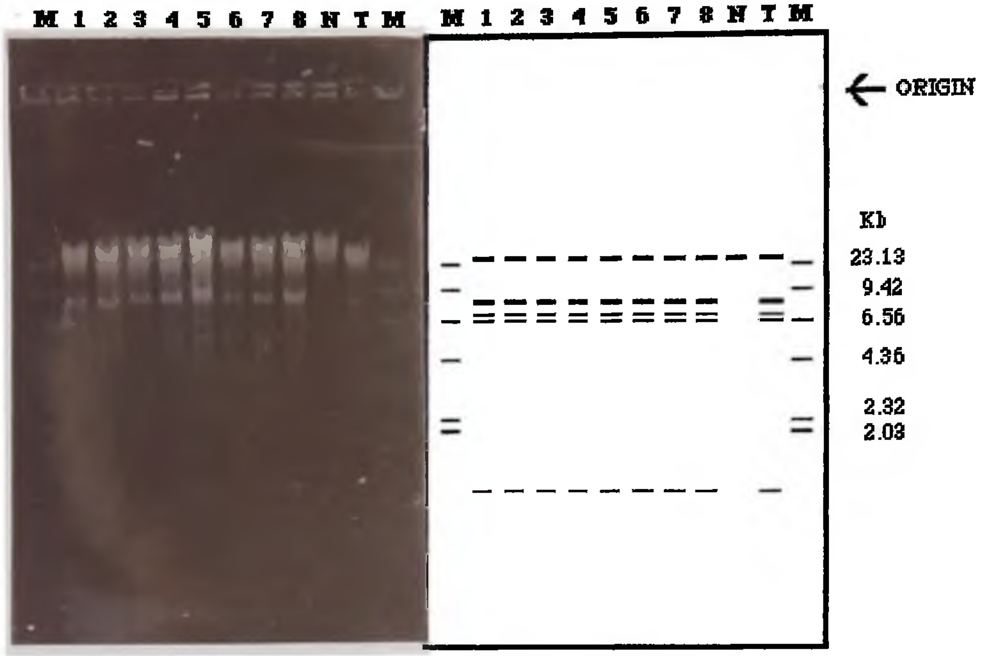
Purified genomic DNA from each isolate was treated with seven restriction endonuclease enzymes separately and each digest was subjected to agarose gel electrophoresis. The total genomic DNA restrictions are shown in Figs. 6A-6G, 6I and 6J. The specific endonuclease digest for all the isolates (with the exception of PW/B/9, PW/B/10a and PW/B/10b) were run side by side in 0.7% agarose gels. Comparison of the patterns was based on the presence or absence of sites (site polymorphism) and on the size variations of the DNA fragments. The results showed the presence of prominent discrete bands in each DNA sample. In some cases, some bands were too faint to permit photographic reproduction.

The *Apa* I restriction pattern (Fig.6A) showed the presence of 1 prominent band of size 8.54 kb and 3 other faint bands of sizes 7.01, 5.87 and 1.30 kb in PW/B/1, PW/B/2 through to PW/B/8, and in PT/B/1. The faint bands can be seen in lane 6. Isolate N/B/1 appeared undigested.

The DNAs appeared uncleaved by *Bam* H I as no strong digested bands were seen (Fig.6B). *Bam* H I cleaves neither in the rDNA nor in the 2-µm plasmid (Philippsen *et al.* 1991).

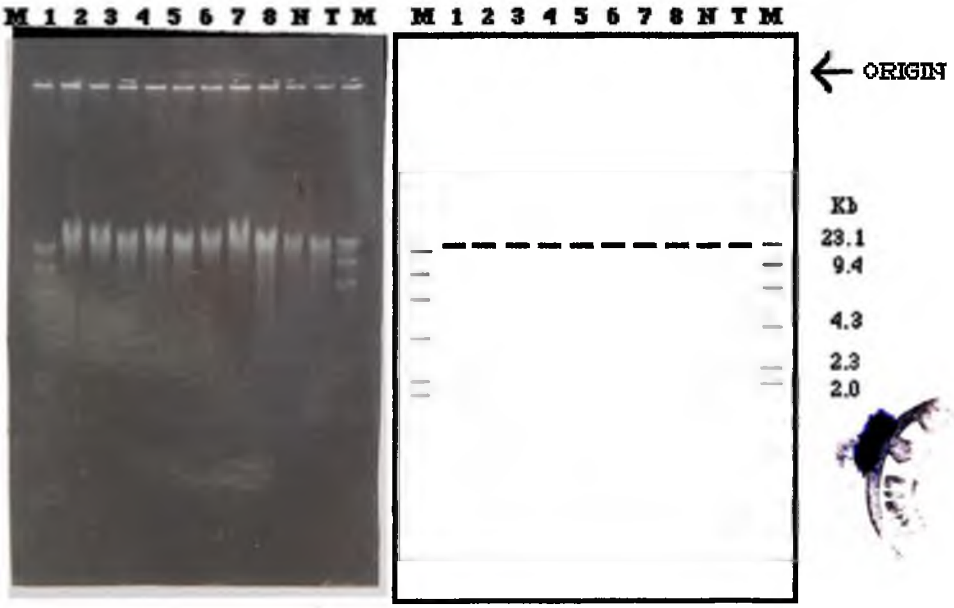
The *Eco* R I spectrum is dominated by rDNA fragments (Philippsen *et al.* 1991) and hence many fragments were generated (Figs.6C and 6I). Three strong bands of sizes 3.18, 2.58 and 2.13 kb are visible in the spectra of PW/B/1, PW/B/2 through to PW/B/8 (Fig. 6C). In PT/B/1, three bands of sizes 4.05, 2.58 and 2.13 kb can be observed (Fig. 6I). In N/B/1 also, 8 bands of sizes 11.33, 9.92, 7.34, 6.18, 3.33, 2.90, 2.42 and 2.23 kb can be seen (Fig. 6I). A number of faint bands were also observed in the spectra of all the isolates.

Two strong bands are seen in the *Hind* III spectra of PW/B/1, PW/B/2 through to PW/B/8 (Fig.6D). The sizes of these bands are 6.93 and 2.70 kb. In PT/B/1, two bands of sizes 6.93 and 2.86 kb can be seen. In N/B/1, two faint bands of sizes 5.89 and 3.0. kb can also be seen. In addition for PW/B/1, PW/B/2 through to PW/B/8, eight bands of sizes 13.89, 11.96, 10.50, 9.36, 8.42, 7.64, 5.06 and 3.03 kb can be seen.



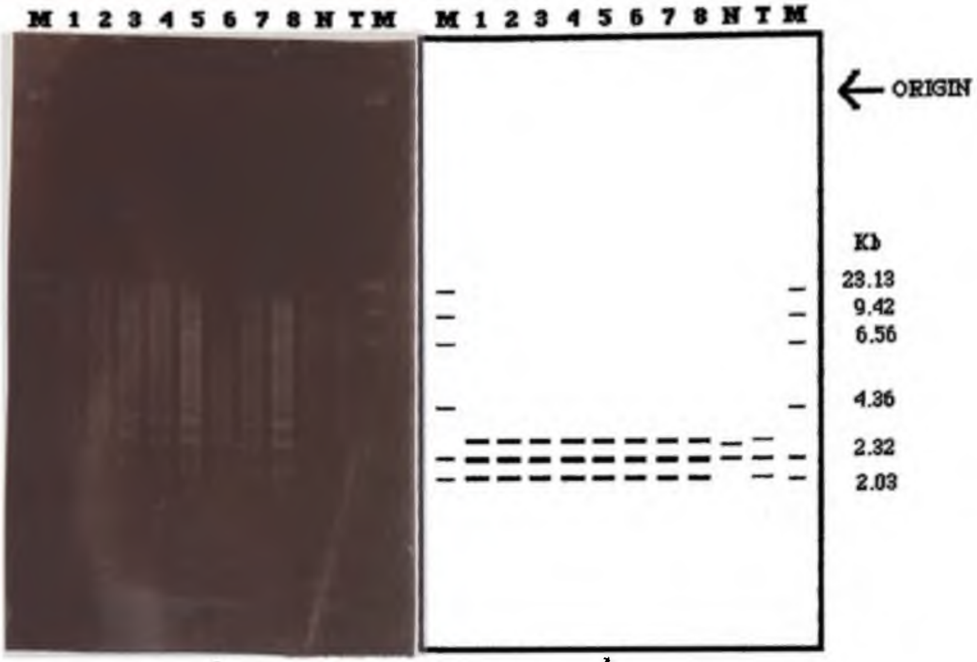
a. b.

Fig. 6A. (a) Restriction fragments pattern of genomic DNA from yeast isolates generated by digestion with *Apa* I. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1
(b) A schematic representation of the gel shown in **a.** (Lines in bold represent strong bands).



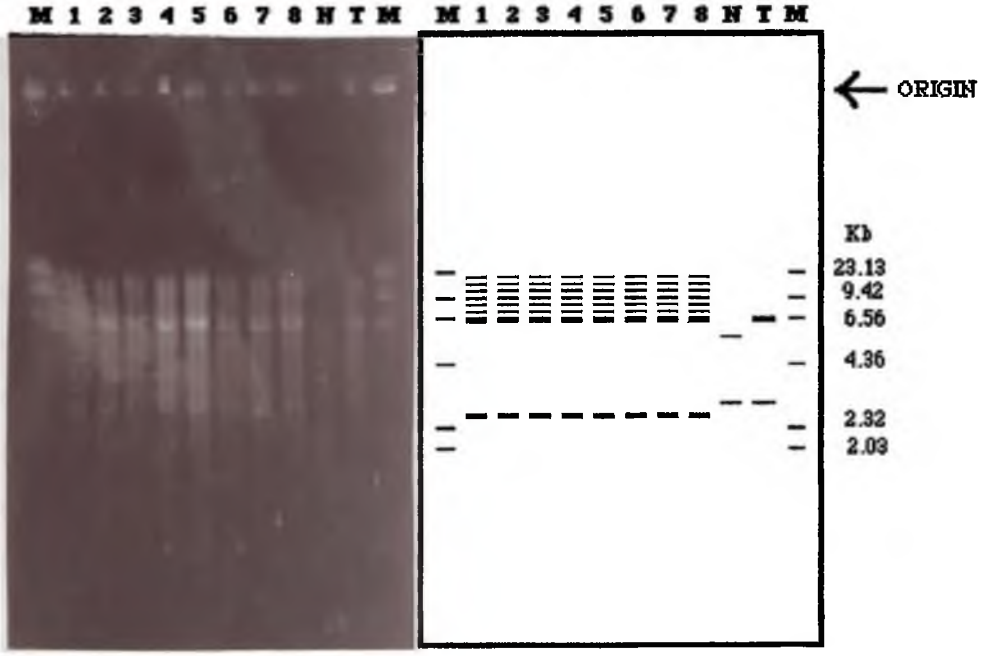
a. b.

Fig. 6B.(a). Restriction fragments pattern of genomic DNA from yeast isolates generated by digestion with *Bam* H I. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1
(b). A schematic representation of the gel shown in a (Lines in bold represent strong bands).



a. b.

Fig. 6C. (a) Restriction fragments pattern of genomic DNA from yeast isolates generated by digestion with *Eco* R I. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1 **(b)** A schematic representation of the gel shown in **a.** (Lines in bold represent strong bands).



a. b.

Fig. 6D. (a) Restriction fragments pattern of genomic DNA from yeast isolates generated by digestion with *Hind* III. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1 **(b)** A schematic representation of the gel shown in **a.** (Lines in bold represent strong bands).

The *Kpn* I spectra showed 1 strong band of size 11.50 kb present in PW/B/1, PW/B/2 through to PW/B/8 but absent in N/B/1 (Fig. 6E). In PT/B/1, two bands of sizes 14.78 and 11.50 kb can be seen. *Kpn* I is known to cut only twice in the rDNA of the yeast genome (Philippsen *et al.*, 1991).

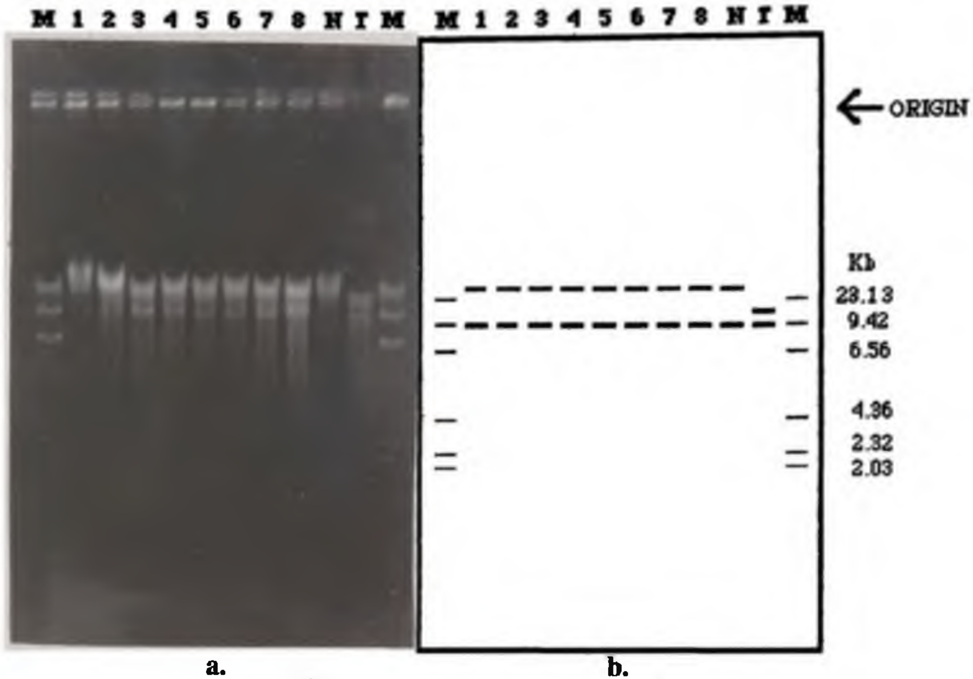
The *Pst* I spectra did not contain any strong digested bands (Fig. 6F) since the enzyme does not cut in the rDNA or in the 2-µm plasmid (Philippsen *et al.*, 1991). Two faint bands of sizes 15.24 and 11.98 kb can be seen in the spectra of PW/B/1, PW/B/2 through to PW/B/8.

The *Sma* I spectra showed a single strong band of size 9.87 kb present in PW/B/1, PW/B/2 through to PW/B/8 and PT/B/1, but absent in N/B/1 (Fig.6G). *Sma* I is also known to cut twice in the rDNA of the yeast genome (Philippsen *et al.*, 1991).

Isolates PW/B/9, PW/B/10a and PW/B/10b, which were later analysed were also subjected to restriction analysis. Purified genomic DNA from these isolates was digested with *Eco* R I and *Hind* III endonuclease enzymes and the electrophoresis run as before. Using isolate PW/B/3 as control, the undigested DNAs and the restriction digests were run side by side. The restriction patterns for PW/B/3 (control) and PW/B/9 were found to be the same for both *Eco* R I (Figs. 6J) and *Hind* III (not shown). The restriction patterns for PW/B/10a and PW/B/10b were also the same for both enzymes but different from those of PW/B/3 and PW/B/9.

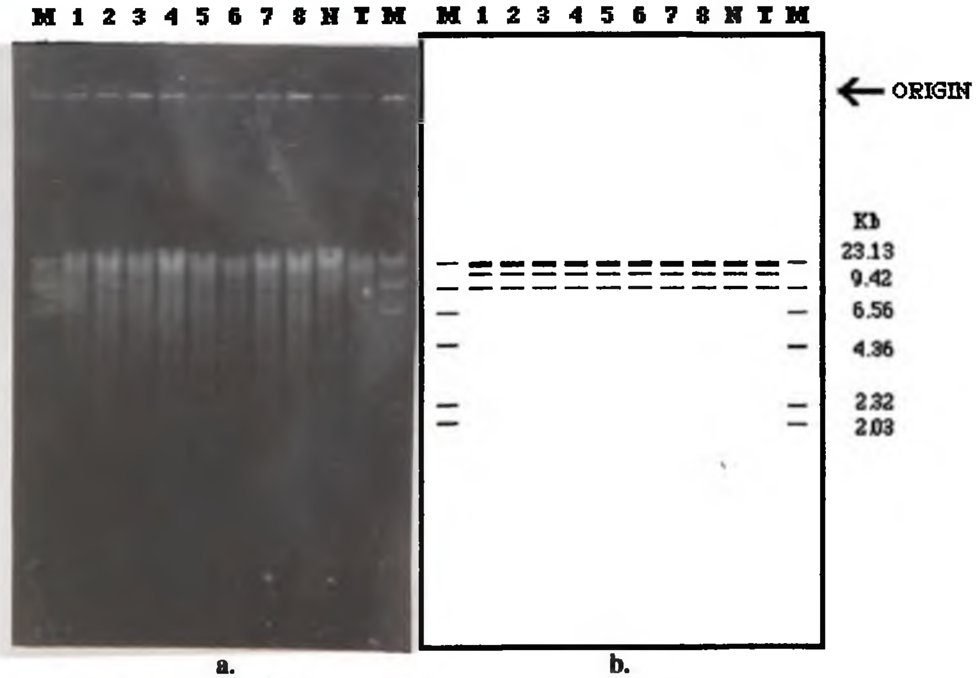
Three strong bands were observed in the *Eco* R I spectrum of PW/B/9 and the sizes of these fragments, 3.30, 2.54 and 2.35 kb were the same as for PW/B/3. Three strong bands were also observed in the spectra of PW/B/10a and PW/B/10b but the sizes of these fragments, 4.00, 3.16 and 2.44 kb were different from those of PW/B/3. The *Hind* III spectra for both PW/B/10a and PW/B/10b also showed no strong bands as observed for PW/B/3 and PW/B/9. The PW/B/3 and PW/B/9 spectra both showed 2 strong bands.

Table 7 presents an inventory of all the differences found thus far, and Tables 8a-8g (see Appendix N) present a catalogue of the restriction fragment sizes of the genomic DNA of all the isolates for each restriction enzyme used.



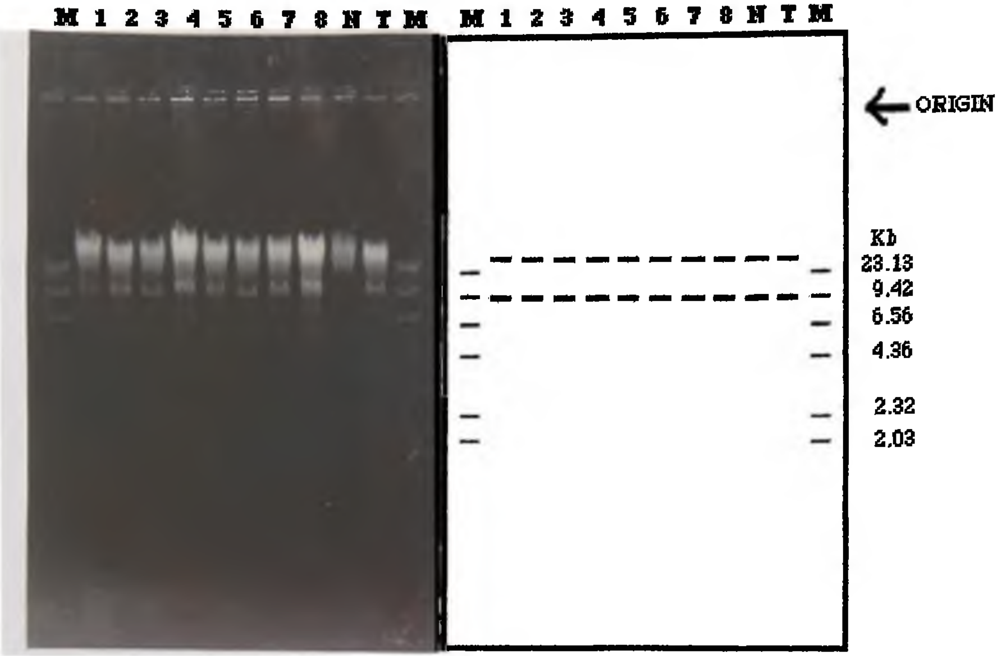
a. b.

Fig. 6E. (a). Restriction fragment patterns of genomic DNA from yeast isolates generated by digestion with *Kpn* I. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1
(b). A schematic representation of the gel shown in **a** (Lines in bold represent strong bands).



a. b.

Fig. 6F. (a). Restriction fragment patterns of genomic DNA from yeast isolates generated by digestion with *Pst* I. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1 **(b.)** A schematic representation of the gel shown in **a** (Lines in bold represent strong bands).



a. b.

Fig. 6G. (a). Restriction fragment patterns of genomic DNA from yeast isolates generated by digestion with *Sma* I. (M) Marker. (1) PW/B/1. (2) PW/B/2. (3) PW/B/3. (4) PW/B/4. (5) PW/B/5. (6) PW/B/6. (7) PW/B/7. (8) PW/B/8. (N) N/B/1. (T) PT/B/1 **(b).** A schematic representation of the gel shown in a. (Lines in bold represent strong bands).

Fig. 6H shows a DNA partial restriction map of the yeast isolates PW/B/1, PW/B/2 through to PW/B/9.

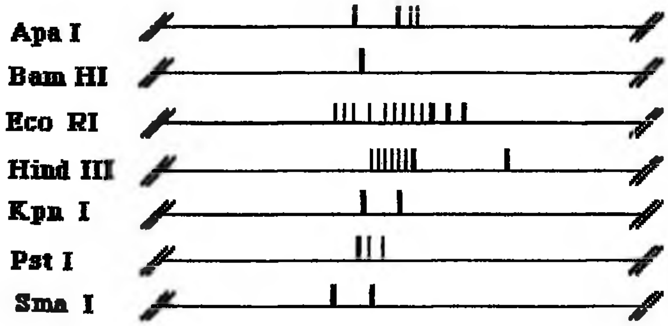


Fig. 6H Palmwine yeast DNA partial restriction map.

Fig I

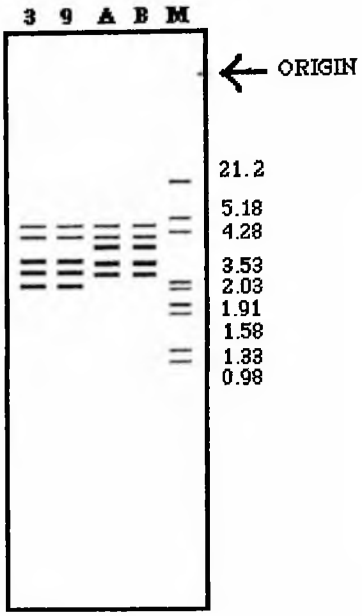


Fig. J

Fig. 6I. A schematic representation showning the comparison of restriction fragment patterns of genomic DNA from palmwine (P), pito (T) and nmeda ` yeast (N) isolates generated by digestion with *Eco* R I. (Lines in bold represent strong bands).

Fig. 6J. A schematic representation showning the comparison of restriction fragment patterns of genomic DNA from PW/B/3 (3), PW/B/9 (9), PW/B/10a (a) and PW/B/10b (b) yeast isolates generated by digestion with *Eco* R I. Marker (M). (Lines in bold represent strong bands).

TABLE.7 SEQUENCE COMPARISON OF GENOMIC DNA OF YEAST ISOLATES.

	POLYMORPHIC RESTRICTION SITE						
SOURCE	*Apa* I	*Bam* H I	*Eco* R I	*Hind* III	*Kpn* I	*Pst* I	*Sma* I
PW/B/1	+	+	+	+	+	+	+
PW/B/2	+	+	+	+	+	+	+
PW/B/3	+	+	+	+	+	+	+
PW/B/4	+	+	+	+	+	+	+
PW/B/5	+	+	+	+	+	+	+
PW/B/6	+	+	+	+	+	+	+
PW/B/7	+	+	+	+	+	+	+
PW/B/8	+	+	+	+	+	+	+
PW/B/9	ND	ND	+	+	ND	ND	ND
PW/B/10a	ND	ND	-	-	ND	ND	ND
PW/B/10b	ND	ND	-	-	ND	ND	ND
N/B/1	-	+	-	-	-	-	-
PT/B/1	+	+	-	-	+	+	+
S288C	ND	+	+	+	ND	ND	ND

KEY: (+) = present ; (-) = absent ; (ND) = not determined

CHAPTER FOUR

4.0 DISCUSSION AND CONCLUSION

The literature is replete with conflicting reports on the number of isolates of yeasts that can be obtained from palmwine. In this study which covered ten different palmwine samples, one sample (from Legon) contained two isolates, while each of the remaining nine samples contained only one yeast isolate. Okafor (1972), found that out of 8 palmwine samples from *Elaies* wine, 7 had single yeast isolates while one sample contained 2 isolates. Okraku-Ofei (1968) and Brown (1990) each reported a single isolate from one palmwine sample. On the other hand, Godwyll (unpublished data) and Owusu (1987) obtained 4 isolates and 5 isolates respectively, from single samples of palmwine. Other workers who have reported the isolation of more than one isolate from palmwine samples (Bassir, 1962 ; Van Pee and Swings, 1971; Faparusi and Bassir, 1972) failed to report the number of samples they studied. It is thus difficult to make a categorical statement about the number of yeast isolates that one may routinely discover from palmwine samples, although it appears that palmwine contains much fewer yeast isolates than the number reported in the past by some authors.

In order to classify or name the yeast isolates, it was first necessary to establish the genus to which they belonged. The properties of the yeasts which were examined to determine the genus were morphological characteristics, and asexual and sexual reproduction. Following the key of Kreger-van Rij (1987) all the palmwine yeasts, and the pito yeast were identified as belonging to the genus *Saccharomyces* . The nmeda yeast was identified as belonging to the genus *Kloeckera*.

Once the genera were known, the species identity could be determined by the established set of physiological and biochemical tests. Beyond the generic level, all the palmwine isolates fit into the description for the species, *cerevisiae*, using the key of Yarrow (1987). The nmeda yeast fits into the description of the species *apis*, using the key of Smith (1987). It is significant that the bulk of palmwine yeasts studied in this work belong to one genus and species. Various workers have reported the isolation of different species of *Saccharomyces* yeasts from palmwine samples (Guilliermond, 1914;

Van Pee and Swings, 1971; Okafor, 1972). All these species, with the exception of *S. exiguus*, have now been reduced to synonymy with *S. cerevisiae* Meyen ex Hansen as a result of the instability of the characteristics by which they were identified and the absence of reproducible isolation (Yarrow, 1987). It is thus possible that the previous reports on the different species of *Saccharomyces* yeasts from palmwine could have been due to the unreliability of the method of speciation that was exclusively used. The only-species that has been identified by earlier workers from this lab on Ghanaian palmwine was *S. cerevisiae* (Owusu, 1987; Brown, 1990; Oduro and Bede, unpublished data). Earlier reports by other workers on the species identity of palmwine yeasts in Ghana also made mention of only the isolation of *S. cerevisiae* (Okraku-Ofei, 1968; Godwyll, unpublished data).

It is possible the only members of the species isolated in this study, mainly *cerevisiae,* have become adapted to growth in the special conditions of palm sap produced in Ghana and the unique method of palmwine tapping adopted in Ghana. Such an adaptation could have occurred as a result of the cultural practices of the palmwine tappers. The methods of palmwine tapping and collection of palm sap have been shown to influence the microbial constituents of the sap (Naghski and Willitis, 1953). The widely practised method of tapping from the felled palm in this country is different from that practised in other West African countries. Moreover, the tapping from the "matured" felled palm results in a different composition of palm sap from that obtained from live trees (Sodah and Matthew, 1971). The evidence suggests that the different methods of tapping the palm tree, coupled with the different cultural practices of palmwine tappers are responsible for the microflora differences found in different palmwine samples.

It is also significant that contrary to reports by other workers (see, for example,Bassir, 1962; Van Pee and Swings, 1971; Okafor 1972; Fahwehinmi, 1981) no yeasts of any other genera, apart from *Saccharomyces* were obtained. The assertion by Ahmad *et al.* (1954) that the 3 yeasts isolated by them *Schizosaccharomyces pombe, Saccharomyces chevalieri* and *Saccharomycodes ludwigii* from the fermenting palm juice of the Palmyra palm (*Brosassus flabellifer*) is world-wide in their association with the natural fermentation of the palm juices seems not applicable to this country. It is suggested that the method used in the treatment of samples and the isolation of yeasts from these samples could have affected the microflora observed.

Although the number of isolates in this study was comparatively small it is possible to draw some general conclusions from the results. *Saccharomyces* species were isolated from palmwine obtained from localities separated, in some cases, by as much as 200 km, and in other cases, by no more than a few kilometres (see Fig. 1). The distribution of the palmwine yeasts in this study, therefore, did not seem to have been influenced by the locality from which the palmwine was obtained. Moreover, as can be seen in Table 5, the yeasts possess different fermentative properties and consequently the by-products of their metabolism could lead to the differences observed in the quality (taste and smell) of palmwine produced in the different localities.

Restriction Fragment Length Polymorphism (RFLP) has been proposed as a taxonomic aid for the study of many microorganisms, and it was interesting to evaluate the contribution of this tool to the identification of palmwine yeasts. In this study, a variety of patterns was observed depending on the restriction enzyme used to cleave the DNA, and these patterns were of taxonomic significance in determining the relatedness between the yeast isolates. Palmwine isolates PW/B/1, PW/B/2 through to PW/B/8 were observed to have the same RFLP patterns for all the restriction enzymes used. These were different from those of N/B/1 from nmeda. Isolate PT/B/1 from pito had RFLP patterns similar to those of PW/B/1, PW/B/2 through to PW/B/8, except for the *Eco*R I and *Hind* III patterns (see Figs. 6A-6G). Isolates PW/B/10a and PW/B/10b had the same *Eco* R I and *Hind* III patterns, but these were different from those of all the other isolates. Isolate PW/B/9, on the otherhand, had *Eco*R I and *Hind* III patterns similar to those of PW/B/1, PW/B/2 through to PW/B/8.

As no restriction site polymorphisms in the repeated DNAs of PW/B/1, PW/B/2 through to PW/B/8 could be detected (see Figs. 6A-6G), it implied that the DNAs had come from the same species. These results confirm the results obtained using the method of phenotypic discontinuity. PW/B/9, which was later analysed, was also shown to be of the same species identity as PW/B/1, PW/B/2 through to PW/B/8, by comparison of its restriction fragment pattern with that of PW/B/3 which was used as a control (see Fig. 6I). Isolates PT/B/1, PW/B/10a and PW/B/10b which had the same phenotypic characteristics, could easily be differentiated using the *Eco* R I and *Hind* III digestions (see Figs. 6H and 6I). It is significant to note that, although PW/B/10a and PW/B/10b had different cell shapes and growth curves, their RFLP patterns were the same, thus suggesting that they are the same species.

Of the 7 restriction enzymes used, *Eco*R I and *Hind* III proved to be the most effective as shown by the number and sizes of fragments generated. These made them useful in the identification of regions of high sequence divergence in the rDNA. Cleavage with *Eco* R I and *Hind* III yielded a variety of patterns that indicated site polymorphisms and hence DNA fragment size variations among samples PW/B/1, PW/B/2 through to PW/B/9, PT/B/1 and isolates PW/B/10a and PW/B/10b. These size variations could be due to insertions or deletions or inversions of nucleotide sequences in the genomic DNA. These affect restriction sites in repeated DNA, and in addition repeated sequences not essential for the life cycle (2-µm plasmid, Ty elements and Y' sequences) may be absent. Isolates PW/B/1, PW/B/2 through to PW/B/9, from this study, can be described as genomic species (that is, species defined by DNA relatedness). Isolates PW/B/10a and PW/B/10b, likewise, can be described as genomic species. Thus PW/B/1, PW/B/2 through to PW/B/9, PW/B/10a and PW/B/10b, and PT/B/1 can be said to be strains of the single species, *Saccharomyces cerevisiae,* since strains may have many variations in property, which, while not affecting nomenclature, are nevertheless of great technical importance. In addition to their taxonomic relevance, DNA gene restriction patterns could be used for strain typing of palmwine yeasts.

A comparison of the DNA restriction patterns of PW/B/1, PW/B/2 through to PW/B/9 with those of *Saccharomyces cerevisiae* strain S288C (the haploid strain often used as a normal laboratory standard) (Sherman, 1991), showed similar RFLP patterns for *Eco*R I,*Hind* III and *Bam*H I (see Table 7). Restriction patterns of *Saccharomyces* species other than strain S288C, are expected to differ from those of other species, due to the expected sequence divergence among the genomes, which also affect restriction sites in repeated DNA. Thus these similarities may indicate a high degree of genomic DNA sequence homology. Southern blotting analysis, Random Amplified Polymorphic DNA (RAPD) PCR and DNA Amplification Fingerprinting (DAF) would enable definite conclusions to be drawn from the RFLP patterns

The primary reason for classifying yeasts is to have an order as well as a name for these microorganisms. This has great value for the palmwine industry. The identification of a yeast at once makes available, from published literature, information on the properties of the yeast and on its previous occurrences. It also allows for the recognition of the same yeast again. Information obtained from DNA fragment size determinations (DNA fingerprinting) after restriction digestions, can be saved permanently by recording the precise size of the DNA fragments. This allows for patterns comparisons across time and space. Thus, this will enable a more consistent and accurate method for characterisation as compared to the instability in the phenotypic characteristics, for isolates obtained from samples collected over a long period of time. As a result of this, the method used to determine the sizes of the DNA restriction fragments is, therefore, very important. The computer programme used for this purpose gave good results. The programme gave information on how well the fitted parabola described the mobility of the calibration standards, as the standard error of fit. The standard error of fit values obtained (see Appendix N), with the exception of that for *Apa* I, were well below the value of 0.5 mm, which has been considered to be normal (Duggleby *et al.,* 1981).

For experiments aimed at quality standardisation of large-scale production and preservation of palmwine, the identity of yeasts used in the fermentation process needs to be properly established. The results obtained from this study show that the main yeast species responsible for palmwine fermentation is *Saccharomyces cerevisiae*. The so-called different yeast species reported in the literature could be sub-species or strains of the *Saccharomyces cerevisiae* yeast.

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APPENDIXES

APPENDIX A

MEDIA FOR THE ISOLATION AND CULTURING OF YEASTS: COMPOSITION AND PREPARATION OF YPD-AGAR AND YPD-BROTH (Sherman, 1991)

Bacto-yeast extract	1.0 g
Bacto-peptone	1.0 g
Dextrose	2.0 g
Bacto-agar	2.0 g
Distilled water	100 ml

The constituents were dissolved in the distilled water in a conical flask; the flask was then plugged with non-absorbent cotton-wool and covered with aluminium foil. The preparation was sterilised by autoclaving for 15 minutes at 121°C and cooled to 45°C. For plate preparations, about 10-15 ml aliquots were transferred into sterile Petri dishes.under aseptic conditions. The Petri dishes were incubated at room temperature (25-28°C) overnight to allow the agar to set and to check for contamination. The Petri dishes were then sealed with cellophane and stored at 4°C.

Broth preparation was done without agar. For the purpose, 4 ml and 40 ml aliquots were dispensed into 10-ml test-tubes and 100-ml volumetric flasks respectively, before autoclaving. Thereafter, the preparations were kept at room temperature until required.

APPENDIX B

MEDIUM FOR MAINTENANCE OF YEASTS: COMPOSITION AND PREPARATION OF MYPD-AGAR SLOPES (Wickerham, 1951; Gilliland, 1981)

Malt extract	0.3 g
Bacto-yeast extract	0.3 g
Bacto-peptone	0.5 g
Dextrose	1.0 g
Bacto-agar	2.0 g
Distilled water	100 ml

The constituents were dissolved in the distilled water in a conical flask by heating. About 8 ml aliquots were transferred into McCartney bottles. The caps were screwed on loosely and the bottles were autoclaved for 15 minutes at 121°C. After autoclaving , the bottles were inclined on a rack so that the agar was just below the neck of the bottles. The screw caps were tightened after 24 hours and the bottles stored at 0-4°C.

APPENDIX C

CYTOLOGICAL METHOD: PREPARATION OF REAGENTS (Collins and Lyne, 1987)

(1) Crystal violet solution: 2.0 g of crystal violet was dissolved in 20 ml of 95% ethanol. 0.8 g of ammonium oxalate was also dissolved in 80 ml of distilled water. The two solutions were mixed and left to stand for 24 hours. The mixture was then filtered and the filtrate was transferred into a dark-brown bottle for storage.

(2) Iodine solution: 2.0 g of potassium iodide and 1.0 g of iodine were ground together in a mortar and dissolved in not more than 20 ml of distilled water. The solution was made up to 100 ml with distilled water when the powdered potassium iodide and iodine had completely dissolved.

(3) Neutral red solution: 0.1 g of neutral red was dissolved in 100 ml of distilled water, followed by the addition of 0.2 ml of 1% (v/v) acetic acid.

APPENDIX D

MEDIUM FOR FORMATION OF PSEUDOMYCELIUM AND TRUE MYCELIUM (COMPOSITION AND PREPARATION) (van der Walt and Yarrow, 1987)

Potato-glucose agar (Fluka, Chemie AG)

Potato extract	4.0 g/L
Dextrose	20.0 g/L
Agar	15.0 g/L

pH 5.6 ± 0.2 at 25°C

In 150 ml of distilled water, 5.85 g of the powder was suspended. The suspension was mixed thoroughly, boiled to dissolve completely and autoclaved at 121°C for 15 minutes. The medium was cooled to about 45°C and about 10-15 ml aliquots poured into sterile Petri-dishes. The Petri-dishes were incubated at 25-28°C for 1-2 days to allow their surfaces to dry. Petri-dishes that were not used after the incubation period were kept at 4°C.

APPENDIX E

(A) MEDIA FOR ASCOSPORE FORMATION.(COMPOSITION AND PREPARATION) (Sherman, 1991)

(1) Pre-sporulation medium

Bacto-yeast extract	0.8 g
Bacto-peptone	0.3 g
Dextrose	10.0 g
Bacto-agar	2.0 g
Distilled water	100 ml

(2) Sporulation medium

Potassium acetate	1.0 g
Bacto-yeast extract	0.1 g
Dextrose	0.05 g
Bacto-agar	2.0 g
Distilled water	100 ml

The two media were prepared separately. The constituents were dissolved in the distilled water, sterilised by autoclaving and dispensed as described under Appendix A.

(B) STAINS FOR ASCOSPORE FORMATION. PREPARATION OF REAGENTS (Schaeffer and Fulton, 1933).

(1) Malachite green (5%, w/v): 2.5 g of crystals was dissolved in 50 ml of distilled water to prepare a 5% aqueous solution. The solution was kept in a dark brown bottle.

(2) Safranine (0.5%, w/v): 0.175 g of safranine was ground in a mortar with 10 ml of 95% ethanol. This was then made up to 100 ml with distilled water to prepare a 0.5% solution.

APPENDIX F

MEDIA FOR PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS (COMPOSITION AND PREPARATION)

(1) Fermentation Basal Medium (Wickerham, 1951).

Bacto-yeast extract	4.5 g
Bacto-peptone	7.5 g
Distilled water	1000 ml

The constituents were dissolved in the distilled water and a few drops of bromothymol blue solution (4 mg/ml) added to give a sufficiently dense green colour. Aliquots (2 ml) were placed in 150 x 12 mm tubes carrying insert tubes. The tubes were sterilised by autoclaving for 15 minutes at 121°C. When cooled, 1 ml concentrated, filter-sterilised sugar solutions (6%, w/v) were added aseptically.

(2) Basal medium for nitrogen auxanographic test (Lodder and Kreger-van Rij, 1952)

Glucose	2.0 g
Potassium dihydrogen phosphate	0.1 g
Magnesium sulphate heptahydrate	0.05 g
Oxoid agar No. 1	2.0 g
Distilled water	100 ml

The constituents were dissolved in the distilled water and heated till a clear solution was obtained. Aliquots (20 ml) were then distributed into screw-capped test tubes and sterilised by autoclaving at 115°C for 15 minutes. After the medium had been cooled to 45°C, a drop of 100-fold concentrated vitamin solution was added to each tube aseptically

(3) Composition of nitrogen sources. (Wickerham, 1946, 1951).

Ammonium sulphate	3.5 g
Potassium nitrate	0.78 g
Tri-ethylamine hydrochloride	0.64 g
L-lysine	0.56 g
Sodium nitrite	0.26 g

(4) Stock concentrated vitamin solution (van der Walt and Yarrow, 1987)

Biotin	0.2 mg
Calcium pantothenate	40.0 mg
Folic acid	0.2 mg
Inositol	200.0 mg
Niacin	40.0 mg
P-aminobenzoic acid	20.0 mg
Pyridozine hydrochloride	40.0 mg
Riboflavin	20.0 mg
Thiamine	100.0 mg

The constituents were dissolved in 1000 ml distilled water and sterilised by filtration. The sterile solution was dispensed in aliquots and stored at -20°C

APPENDIX G

TURBIDIMETRIC MEASUREMENTS.PREPARATION OF FORMALDEHYDE-SALINE SOLUTION (Pringle and Moor, 1975).

(1) Formaldehyde solution (40%, v/v): 40 ml of stock formaldehyde solution was dispensed into a 100-ml volumetric flask and made up to the mark with distilled water.

(2) Saline solution (0.9%, w/v): 0.23 g of NaCl crystals was dissolved in 20 ml of distilled water. The volume was then adjusted to 25 ml with distilled water and the solution sterilised by autoclaving at 121°C for 15 minutes.

To prepare formaldehyde-saline solution, 37 ml of the 40% formaldehyde solution was mixed with 6.3 ml of the 0.9% saline solution. The solution was stored at room temperature.

APPENDIX H

CELL COUNTING (WHO/LAB/88.3, 1988)

The Improved Neubauer Chamber.

The counting chamber of the improved Neubauer chamber consists of a depressed area of a glass slide which is converted to a volumetric chamber when overlaid by a cover glass.

The ruled area of the counting chamber is 3 x 3 mm giving 9 large squares each of 1 x 1 mm. The depth of the chamber with the cover glass in position is 0.1 mm. Therefore, individually, each of the large squares has a volume of 0.1 μ l.

The central 1 x 1 mm area consists of 25 groups (0.2 x 0.2 mm) of 16 squares separated closely by ruled triple lines. For the yeast cell count, it was sufficient to use five 0.2 x 0.2 mm groups marked by "P" (Fig. 7B), which are together equivalent to a volume of 0.02 μl in area A (Fig. 7A). This was repeated for area B and the average of the 2 counts was used in the calculations.

Sample Calculation of Cell Number.

The number of cells was calculated using the formula ;

$$\text{Number of cells/ml} = \frac{\text{Number of cells counted (N)}}{\text{Volume counted } (\mu\text{l})} \times \text{Dilution Factor} \times 10^3$$

$$\text{Therefore, yeast cell count/ml} = \frac{N}{0.02} \times 10 \times 10^3$$

$$= N \times 5 \times 10^5 \text{ cells/ml}$$

For example, after 2 hours, N for cells in area A = 29

$$\text{Therefore, yeast cell count /ml} = 29 \times 5 \times 10^5$$

$$= 1.45 \times 10^7 \text{ cells/ml}$$

Statistical Evaluation of Obtained Results.

The standard deviation (SD) of the count is approximately the square root of the count and the coefficient of variation (CV) is obtained by;

$$\frac{SD}{MEAN} \times 100\%$$

95% of the results will lie within ± 2 CV of the true value.

For example, for the 29 cells counted as above, yeast cell count = 1.45 x 10^7 cells/ml

$$\text{Therefore, SD} = \sqrt{29} = 5.39$$

$$CV = \frac{5.39 \times 100}{29} = 18.59$$

$$\text{Yeast cell count} \times 2CV = 2 \times \frac{18.59}{100} \times (1.45 \times 10^7/\text{ml})$$

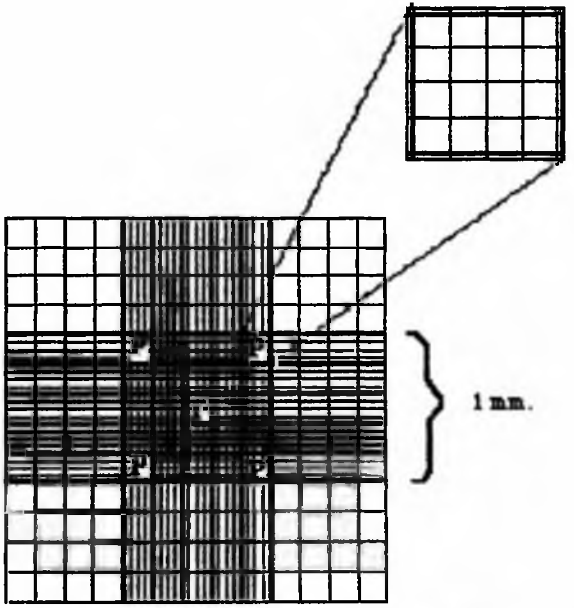

FIG.7B IMPROVED NEUBAUER COUNTING CHAMBER.

FIG. 7 THE NEUBAUER COUNTING CHAMBER.

$$= 0.54 \times 10^{7}\ /\text{ml}$$

$$\text{Yeast cell count} \pm 2\text{CV} = (1.45 \pm 0.54) \times 10^{7}/\text{ml}$$

$$= 0.91\text{-}1.90 \times 10^{7}/\text{ml}$$

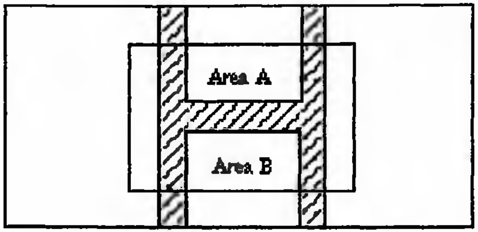

FIG. 7A DESIGN OF COUNTING CHAMBER.

APPENDIX I

SPHAEROPLAST PREPARATION: PREPARATION OF REAGENTS.

(1) Potassium phosphate buffer, 0.05 M, pH 7.5: Stock solutions of KH_2PO_4 and K_2HPO_4, each of 1 M concentrations, were prepared.

1 M KH_2PO_4: 13.609 g of crystals was dissolved in 100 ml of distilled water.

1 M K_2HPO_4: 17.418 g of crystals was dissolved in 100 ml of distilled water.

The buffer was prepared by combining 0.67 ml of KH_2PO_4 and 4.33 ml of K_2HPO_4 and diluting to 100 ml with distilled water. The pH was calculated according to the Henderson-Hasselbach equation;

$$pH = pk' + \left[\frac{\text{Proton Acceptor}}{\text{Proton Donor}}\right]$$

where pk' = 6.86 at 25°C

(2) 1.2 M sorbitol: 10.930 g of sorbitol was dissolved in 50 ml of distilled water and sterilised by autoclaving at 121°C for 15 minutes.

(3) Enzyme solution (Lyticase from *Arthrobacter luteus,* Sigma L 8137): The partially purified lyophilized powder (50,000 units) was dissolved in 10 ml of potassium phosphate buffer to give 500 units/ml of lyticase stock solution. A working solution of 100 units/ml was prepared by dilution of the enzyme stock solution when needed. All the solutions were stored at -20°C.

APPENDIX J

ISOLATION AND PURIFICATION OF DNA: PREPARATION OF REAGENTS.

(1) 0.5 M EDTA stock solution, pH 8.0: To 46.53 g of disodium ethylene diaminetetra-acetate.$2H_2O$ was added 200 ml of distilled water. This was vigorously stirred on a magnetic stirrer as the pH was adjusted to 8.0 with the addition of NaOH crystals. After the pH adjustment, the volume was made up to 250 ml with distilled water. The solution was dispensed into aliquots and sterilised by autoclaving at 121°C for 15 minutes.

(2) 10% (w/v) sodium dodecyl sulphate (SDS): 10 g of SDS was dissolved in 90 ml of distilled water. This was heated to 68°C. The pH was adjusted to 7.2 by the

addition of a few drops of concentrated HCl and the volume made up to 100 ml with distilled water.

(3) 5 M potassium acetate: 24.535 g of potassium acetate was dissolved in 50 ml of distilled water and the solution kept at -20°C.

(4) 0.5 M Tris-HCl stock solution , pH 7.4: 30.275 g of Tris base was dissolved in 400 ml of distilled water. The pH was adjusted to 7.4 by the addition of a few drops of concentrated HCl. The solution was allowed to cool to room temperature before the final adjustments to the pH were made. The volume was then adjusted to 500 ml with distilled water and the solution dispensed into aliquots and sterilised by autoclaving at 121°C for 15 minutes.

(5) TE buffer (pH 7.4), 10 mM Tris-Cl, pH 7.4; 1 mM EDTA, pH 8.0: The buffer was prepared by combining 10 ml of the Tris-Cl stock solution and 1 ml of the EDTA stock solution and diluting to 500 ml with distilled water. The solution was dispensed into aliquots, autoclaved (121°C for 15 minutes) and kept at room temperature.

(6) TE buffer (pH 7.4), 20 mM Tris-Cl, pH 7.4; 1 mM EDTA, pH 8.0: This was prepared as above using 20 ml of the Tris-Cl stock solution.

(7) 50 mM EDTA, pH 8.5: To 5.0 ml of stock EDTA solution was added 35 ml of distilled water. The pH was adjusted to 8.5 by the addition of NaOH (1 M) and the volume made up to 50 ml with distilled water. The solution was sterilised by autoclaving at 121°C for 15 minutes and kept at room temperature.

(8) Bovine pancreas RNase, 10 mg/ml: Pancreatic ribonuclease A (Sigma R9009) was dissolved in 10 mM Tris-Cl, pH 7.5, 15 mM NaCl at a concentration of 10 mg/ml in a polypropylene container. This stock solution was placed in a boiling water bath and removed after exactly 5 minutes; it was allowed to equilibrate to room temperature and aliquots were dispensed into tubes which were stored at -20°C.

(9) TES buffer, 0.05 M Tris-Cl, pH 8.0; 0.05 M EDTA, pH 8.0; 0.1M NaCl: The buffer was prepared by combining 2.5 ml of Tris-Cl stock solution (5 M, pH 8.0), 2.5 ml of EDTA stock solution (5 M, pH 8.0) and 1.46 g of NaCl. The volume was made up to

250 ml with distilled water. The solution was sterilised by autoclaving at 121°C for 15 minutes and kept at room temperature.

(10) Buffer-saturated phenol (Grimont and Grimont, 1991): To 100 g of phenol was added 100 ml of TES buffer and 0.01 g of 8-hydroxyquinoline. The mixture was heated in a water bath at 50-60°C to dissolve the phenol. The liquified phenol was stirred for 5 minutes at low speed with a magnetic stirrer and stored at 4°C. Before use, the buffer-saturated phenol was shaken and poured into a separation funnel, the phases were allowed to separate and the coloured phenol phase collected.

(11) Chloroform/isoamyalcohol mixture (24:1, v/v): 24 volumes of chloroform was mixed with 1 volume of isoamyalcohol and the mixture stored at 4°C.

(12) Phenol/chloroform/isoamyalcohol mixture (25:24:1, v/v/v): An equal volume of buffer-saturated phenol was mixed with an equal volume of chloroform/isoamyalcohol mixture and stored at 4°C in a dark bottle.

APPENDIX K

RESTRICTION ENDONUCLEASE DIGESTION: COMPOSITION AND PREPARATION OF REAGENTS.

(1) Restriction Enzyme Stock Solutions.

RESTRICTION ENDONUCLEASE	PROKARYOTIC SOURCE	RECOGNITION SEQUENCE	ACTIVITY (Units/µl)
Apa **I**	*Acetobacter pasteurianus*	5'-GGGCC/C-3'	10
Bam **H I**	*Bacillus amyloliquefaciens*	5'-G/GATCC-3'	10
Eco **R I**	*Escherichia coli*	5'-G/AATTC-3'	10
Hind **III**	*Haemophilus influenzae Rd*	5'-A/AGCTT-3'	10
Kpn **I**	*Klebsiella pneumoniae*	5'-GGTAC/C-3'	9
Pst **I**	*Providencia stuartii*	5'-CTGCA/G-3'	12
Sma I	*Serratia marcescens* S_b	5'-CCC/GGC-3'	11

The enzymes were stored at -20°C. With the exception of the *Eco* R I, which was obtained from BRL (Bethesda Research Laboratories), the rest were obtained from Boehringer.

(2) Restriction Enzyme Digestion Buffers: The buffers were supplied by the manufacturers, each enzyme with its specific buffer and at concentrations which were 10x the final concentrations. The buffers were stored at -20°C.

For the *Eco*R I digestion, the assay buffer was supplemented with bovine serum albumin (100x concentration, BRL) to a final concentration of 0.1 mg/ml.

(3) Gel-loading Buffer (Sigma G-2526):

0.05% (w/v) bromophenol blue
40% (w/v) sucrose
0.1 M EDTA, pH 8.0
0.5% (w/v) sodium lauryl sulphate (SDS).

One volume of gel-loading buffer was added to 1-4 volumes of sample, and mixed well before loading.

APPENDIX L

REAGENTS FOR ELECTROPHORESIS.

(1) Electrophoresis buffer, Tris-borate (TBE), concentrated stock solution: 54 g of Tris base and 27.5 g of boric acid were dissolved in 900 ml of distilled water. 20 ml of 0.5 M EDTA (pH 8.0) was added and the volume made up to 1000 ml with distilled water. The 5x solution was dispensed into aliquots and stored at room temperature.

Agarose gel electrophoresis was carried out with a 1:10 dilution of the concentrated stock solution to prepare a 0.5x working solution.

(2) Ethidium bromide, 10 mg/ml: 0.1 g of ethidium bromide was added to 10 ml of water and stirred on a magnetic stirrer for several hours to ensure that the dye had dissolved. The container was wrapped in aluminium foil and stored at room temperature.

Gloves were worn when working with solutions that contained this dye.

(3) Nucleic acid markers, Lambda DNA *Hind* III Digest (Sigma D4521) and Lambda DNA *Eco*R I *Hind* III Digest (Sigma D3398): The 0.25 lyophilized powders were dissolved in 50 ml of sterile distilled water to give a stock solution. The working solution was made by preparing a 1 in 4 dilution of the stock solution; followed by the

addition of 1 volume of gel-loading buffer to 4 volumes of the working solution. All the solutions were stored at -20°C.

The sizes (bp) of the fragments are as follows:

(a) Lambda DNA *Hind* III Digest (Sigma D4521) ; 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564 and 125.

(b) Lambda DNA *Eco*R I *Hind* III Digest (Sigma D3398) ; 21,226, 5,184, 4,973, 4,277, 3,530, 2,027, 1,907, 1,584, 1,330, 983, 831, 564 and 125.

APPENDIX M

COMPUTER PROGRAMME FOR THE ANALYSIS OF THE SIZE OF RESTRICTION FRAGMENTS OF DNA

```
1000 REM *** THE FOLLOWING ARRAYS WERE USED
1010 REM ***      S - SIZE OF FRAGMENT
1020 REM ***      M - MOBILITY OF FRAGMENT
1030 REM *** THE FOLLOWING STRINGS ARE USED
1040 REM ***      L$- "Y" OR "N" DEPENDING ON ANSWER TO "LAMBDA" QUESTION
1050 REM ***      S$-"KB", "MD" OR "ST" (IF OTHER STANDARDS USED)
1060 REM ***      R$-REPLY TO QUESTION Y/N OR S/C/N
1070 REM *** THE FOLLOWING SCALARS ARE USED
1080 REM ***      Z - TEMPORARY STORAGE FOR ARRAY ELEMENTS
1090 REM ***      Z1 - TEMPORARY STORAGE FOR SIGMA (F - M (I))
1100 REM ***      S1- SIGMA S AND MEAN S
1110 REM ***      S2 - SIGMA S^2 AND MEAN S^2
1120 REM ***      M1- SIGMA M AND MEAN M
1130 REM ***      M2 - A MOBILITY FOR WHICH A SIZE WILL BE CALCULATED
1140 REM ***      F - A QUADRATIC FUNCTION VALUE
1150 REM ***      R - RESIDUAL SUM OF SQUARES
1160 REM ***      I - LOOP VARIABLE AND TEMPORARY VARIABLE
1170 REM ***      D - TEMPORARY VARIABLE
1180 REM ***      N1 - THE NUMBER OF STANDARDS
1190 REM ***      N - ARRAY INDEX IN CORRECTION LOOP
1200 REM ***      Y - FACTOR USED IN MD AND KB CONVERSION
1210 REM ***      D3 - TEMPORARY VARIABLE
1220 REM ***      Q1)
1230 REM ***      Q2) - QUADRATIC COEFFS.
1240 REM ***      Q3)
1250 REM ***      R1, R2, A2, A3, A4, P1, P2 ARE USED IN THE QUADRATIC REGRESSION
1260 REM ***
1270 REM **********************************************************************
1280 REM DIMENSION ARRAYS FOR UP TO 20 STANDARDS
1290 DIM S(20), M(20)
1300 REM  *** DATA FOR LAMBDA-ECO + LAMBDA-HIND STANDARDS [IN MD]
1310 DATA 15.3, 13.7, 6.12, 4.68, 3.70, 3.56, 3.03, 2.76, 2.09, 1.46, 1.20
1320 PRINT
1330 PRINT "PARABOLIC STANDARD CURVE FOR DNA GELS"
1340 PRINT
1350 PRINT "HAVE YOU USED LAMBDA-ECO + LAMBDA-HIND AS STANDARDS (Y/N)":
1360 INPUT L$
1370 IF L$ = "N" GO TO 1650
1380 IF L$ < > "Y" GO TO 1350
1390 PRINT "DO YOU WANT SIZES IN MD OR KB":
1400 INPUT S$
1410 REM *** CHECK REPLY - MUST BE EITHER "MD" OR "KB"
```

```
1420 IF S$ = "MD" THEN 1440
1430 IF S$ < > "KB" THEN 1390
1440 PRINT
1450 PRINT " N      "; S$ : "    MOBILITY"
1460 REM *** SET Y = 1 IF SIZES IN MD ELSE SET Y = 1.545 IF SIZES IN KB
1470 REM *** NOTE THAT AT THIS STAGE S$ IS EITHER "KB" OR "MD"
1480 Y = 1
1490 IF S$ = "KB" THEN Y = 1.545
1500 REM *** RESTORE POINTER TO BEGINNING OF DATA STATEMENT THEN
1510 REM *** READ STANDARDS STORED IN DATA STATEMENT, SCALE WITH
1520 REM *** Y AND INPUT MOBILITY FROM KEYBOARD
1530 RESTORE
1540 FOR I = 1 TO 12
1550 READ S (I)
1560 S(I) = S (I) * Y
1570 PRINT  I; S (I):
1580 INPUT  M (I)
1590 NEXT  I
1600 N1 = 12
1610 REM *** GO TO EDIT INPUT DATA
1620 GO TO 1780
1630 REM *** INPUT NUMBER OF STANDARDS AND THE SIZE AND MOBILITY
1640 REM *** FOR EACH STANDARD
1650 S$ = "ST"
1660 PRINT
1670 PRINT "NUMBER OF STANDARDS  = ";
1680 INPUT N1
1690 PRINT
1700 PRINT "  N       SIZE, MOBILITY"
1710 PRINT
1720 FOR I = 1 TO N1
1730 PRINT I;
1740 INPUT S (I), M (I)
1750 NEXT I
1760 REM *** [OPTIONALLY] LIST EDIT INPUT DATA - INPUT LINE (N)
1770 REM *** THEN SIZE, MOBILITY OR MOBILITY ONLY, ACCORDING TO L$
1780 PRINT
1790 PRINT "LIST STANDARDS AND MOBILITIES (Y/N)";
1800 INPUT R$
1810 IF R$ = "N" THEN 1780
1820 IF R$ < > "Y" THEN 1790
1830 PRINT
1840 PRINT "  N    "; S$ ;"  MOBILITY"
1850 PRINT
1860 FOR I = 1 TO N1
1870 PRINT  I; S (I); M (I)
1880 NEXT I
1890 PRINT
1900 PRINT
1910 PRINT "ENTER CORRECTIONS; N THEN CORRECTED DATA. END WITH N = 0"
1920 PRINT
1930 PRINT "N";
1940 INPUT N
1950 IF N = 0 GO TO 2050
1960 IF L$ = "Y" GO TO 1990
1970 PRINT "SIZE";
1980 INPUT S (N)
1990 PRINT  "MOBILITY";
2000 INPUT M (N)
2010 PRINT
2020 GO TO 1930
2030 REM *** CALCULATE QUADRATIC REGRESSION COEFFICIENTS
2040 REM *** COEFFS LOCATED IN [X^2]  Q3  [X^1]  Q2  [X^0]  Q1
2050 S1 = 0
```

```
2060 S2 = 0
2070 M1 = 0
2080 FOR I = 1 TO N1
2090 Z = LOG (S (I) )
2100 S1 = S1 + Z
2110 S2 = S2 + Z^2
2120 M1 = M1 + M (I)
2130 NEXT I
2140 S1 = S1 / N1
2150 S2 = S2 / N1
2160 M1 = M1 / N1
2170 A2 = 0
2180 A3 = 0
2190 A4 = 0
2200 P1 = 0
2210 P2 = 0
2220 FOR I = 1 TO N1
2230 Z = LOG (S (I))
2240 R1 = Z - S1
2250 R2 = Z^2 - S2
2260 A2 = A2 + R1^2
2270 A3 = A3 + R1 * R2
2280 A4 = A4 + R2^2
2290 P1 = P1 + R1 * M (I)
2300 P2 = P2 + R1 * M (I)
2310 NEXT I
2320 D = A2 * A4 - A3^2
2330 A2 = A2 / D
2340 A3 = - A3 / D
2350 A4 = A4 / D
2360 Q3 = A3 * P1 + A2 * P2
2370 Q2 = A4 * P1 + A3 * P2
2380 Q1 = M1 - Q2 * S1 - Q3 *S2
2390 REM *** PRINT HEADINGS
2400 PRINT
2410 PRINT "FIT TO THE HEADINGS"
2420 PRINT
2430 PRINT "          MOBILITY                    SIZE "
2440 PRINT " ACTUAL     CALCULATED       ACTUAL     CALCULATED"
2450 REM *** CALCULATE MOBILITY AND SIZE , PRINT OBSERVED AND
2460 REM *** CALCULATED MOBILITY AND OBSERVED AND CALCULATED SIZE.
2470 REM *** CALCULATE AND SUM THE RESIDUALS, AND CALCULATE
2480 REM *** AND PRINT THE STANDARD ERROR OF FIT.
2490 Z1 = 0
2500 FOR I = 1 TO N1
2510 Z = LOG (S (I))
2520 F = Q1 + Q2 * Z + Q3 * Z^2
2530 Z1 = Z1 + (F - M (I))^2
2540 PRINT M (I); F ; S (I);
2550 Z = M (I)
2560 D = Q2^2 - 4 * Q3 (Q1 - Z)
2570 Z = -(SQR (D) + Q2) / (2 * Q3)
2580 PRINT "  ", EXP (Z)
2590 NEXT I
2600 PRINT
2610 PRINT "STD ERR OF FIT = "; SQR (Z1 / (N1 - 3)); "MM"
2620 PRINT
2630 PRINT "ENTER MOBILITY OF UNKNOWNS... END WITH MOBILITY = 0"
2640 PRINT "MOBILITY = ";
2650 INPUT M2
2660 IF M2 = 0 GO TO 2720
2670 D = Q2^2 - 4 * Q3 * (Q1 - M2)
2680 Z = - (SQR (D) +Q2) / (2 *Q3)
2690 PRINT "     SIZE = "; EXP (Z)
```

```
2700 GO TO 2640
2710 REM *** LOGICAL END OF PROGRAMME
2720 PRINT
2730 REM *** ASK IF REUSE OF PROGRAMME REQUIRED EITHER USING
2740 REM *** SAME STANDARDS OR, CORRECTING EXISTING STANDARDS
2750 REM *** OR USING NEW STANDARDS
2760 PRINT
2770 PRINT "REUSE PROGRAMME (Y/N)":
2780 INPUT R$
2790 IF R$ = "N" THEN 2880
2800 IF R$ < > "Y" THEN 2770
2810 PRINT "SAME STANDARDS (S), CORRECT EXISTING STANDARDS (C):
2820 PRINT " OR NEW STANDARDS (N)":
2830 INPUT R$
2840 IF R$ = "S" THEN 2620
2850 IF R$ = "C" THEN 1780
2860 IF R$ = "N" THEN 1350
2870 GO TO 2810
2880 PRINT
2890 PRINT " END OF PROGRAMME"
2900 PRINT
2910 END
```

APPENDIX N

TABLE 8a. CATALOGUE OF RESTRICTION FRAGMENTS: DIGEST WITH *Apa* I

NO. OF FRAGS	PW/B/1	PW/B/2	PW/B/3	PW/B/4	PW/B/5	PW/B/6	PW/B/7	PW/B/8	N/B/1	PT/B/1
	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
1	8.54	8.54	8.54	8.54	8.54	8.54	8.54	8.54	--	8.54
2	7.01	7.01	7.01	7.01	7.01	7.01	7.01	7.01		7.01
3	5.87	5.87	5.87	5.87	5.87	5.87	5.87	5.87		5.87
4	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30		1.30

Standard Error of Fit = 0.9278 mm

TABLE 8b. CATALOGUE OF RESTRICTION FRAGMENTS: DIGEST WITH *Eco* R I

NO. OF FRAGS	PW/B/1	PW/B/2	PW/B/3	PW/B/4	PW/B/5	PW/B/6	PW/B/7	PW/B/8	N/B/1	PT/B/1
	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
1	21.11	21.11	21.11	21.11	21.11	21.11	21.11	21.11	11.33	8.73
2	17.64	17.64	17.64	17.64	17.64	17.64	17.64	17.64	9.92	7.73
3	14.93	14.93	14.93	14.93	14.93	14.93	14.93	14.93	7.34	6.18
4	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	6.18	4.05
5	10.01	10.01	10.01	10.01	10.01	10.01	10.01	10.01	4.72	3.18
6	7.73	7.73	7.73	7.73	7.73	7.73	7.73	7.73	5.05	2.58
7	6.18	6.18	6.18	6.18	6.18	6.18	6.18	6.18	3.33	2.22
8	4.48	4.48	4.48	4.48	4.48	4.48	4.48	4.48	2.90	2.13
9	3.67	3.67	3.67	3.67	3.67	3.67	3.67	3.67	2.42	
10	3.18	3.18	3.18	3.18	3.18	3.18	3.18	3.18	2.23	
11	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58		
12	2.13	2.13	2.13	2.13	2.13	2.13	2.13	2.13		

Standard Error of Fit = 0.2836 mm

TABLE 8c. CATALOGUE OF RESTRICTION FRAGMENTS: DIGEST WITH *Hind* III.

NO. OF FRAGS	PW/B/1	PW/B/2	PW/B/3	PW/B/4	PW/B/5	PW/B/6	PW/B/7	PW/B/8	N/B/1	PT/B/1
	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
1	13.89	13.89	13.89	13.89	13.89	13.89	13.89	13.89	5.89	6.39
2	11.96	11.96	11.96	11.96	11.96	11.96	11.96	11.96	3.03	2.86
3	10.50	10.50	10.50	10.50	10.50	10.50	10.50	10.50		
4	9.36	9.36	9.36	9.36	9.36	9.36	9.36	9.36		
5	8.42	8.42	8.42	8.42	8.42	8.42	8.42	8.42		
6	7.64	7.64	7.64	7.64	7.64	7.64	7.64	7.64		
7	6.39	6.39	6.39	6.39	6.39	6.39	6.39	6.39		
8	5.06	5.06	5.06	5.06	5.06	5.06	5.06	5.06		
9	3.03	3.03	3.03	3.03	3.03	3.03	3.03	3.03		
10	2.70	2.70	2.70	2.70	2.70	2.70	2.70	2.70		

Standard Error of Fit = 0.0911 mm

TABLE 8d CATALOGUE OF RESTRICTION FRAGMENTS: DIGEST WITH *Kpn* I

	PW/B/1	PW/B/2	PW/B/3	PW/B/4	PW/B/5	PW/B/6	PW/B/7	PW/B/8	N/B/1	PT/B/1
NO. OF FRAGS	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
1	17.10	17.10	17.10	17.10	17.10	17.10	17.10	17.10	17.10	14.80
2	11.50	11.50	11.50	11.50	11.50	11.50	11.50	11.50		11.50

Standard Error of Fit = 0.2856 mm

TABLE 8e. CATALOGUE OF RESTRICTION FRAGMENTS: DIGEST WITH *Pst* I

	PW/B/1	PW/B/2	PW/B/3	PW/B/4	PW/B/5	PW/B/6	PW/B/7	PW/B/8	N/B/1	PT/B/1
NO. OF FRAGS	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
1	15.24	15.24	15.24	15.24	15.24	15.24	15.24	15.24		
2	11.98	11.98	11.98	11.98	11.98	11.98	11.98	11.98		

Standard Error of Fit = 0.3481 mm

TABLE 8f. CATALOGUE OF RESTRICTION FRAGMENTS: DIGEST WITH *Sma* I

	PW/B/1	PW/B/2	PW/B/3	PW/B/4	PW/B/5	PW/B/6	PW/B/7	PW/B/8	N/B/1	PT/B/1
NO. OF FRAGS	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb	Kb
1	23.40	23.40	23.40	23.40	23.40	23.40	23.40	23.40	23.40	23.40
2	9.87	9.87	9.87	9.87	9.87	9.87	9.87	9.87		

Standard Error of Fit = 0.0373 mm

TABLE 8g. COMPARISON OF RESTRICTION FRAGMENT SIZES: DIGEST WITH *Eco* R I

	PW/B/3	PW/B/9	PW/B/10a	PW/B/10b
NO. OF FRAGS	Kb	Kb	Kb	Kb
1	5.56	5.56	5.56	5.56
2	4.68	4.68	4.68	4.68
3	3.30	3.30	4.00	4.00
4	2.54	2.54	3.16	3.16
5	2.35	2.35	2.44	2.44

Standard Error of fit = 0.2229 mm