DIVERSITY WITHIN YEAST INVOLVED IN SPONTANEOUS FERMENTATION OF *PITO*

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DECLARATION AND CERTIFICATION

I, Glover, Richard Lander Kwame, hereby declare that this thesis "DIVERSITY WITHIN YEAST INVOLVED IN SPONTANEOUS FERMENTATION OF *PITO*" consists entirely of my own work produced from research undertaken under supervision towards the Doctor of Philosophy (PhD) Biological Sciences Degree and that to the best of my knowledge, it contains no material previously published by another person; nor has it been presented for another degree elsewhere, except for the permissible excepts/references from other sources, which have been duly acknowledged.

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DEDICATION

This work is dedicated to my late parents---Togbe Peter Woamadey Kwasi Glover and Madam Sarah Nanor Doe Kpetigo-Glover, who, though unlettered, struggled to bring me this far.

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ABSTRACT

A survey was conducted in ten (10) "Dagarti *pito"* production sites located in nine towns within eight administrative regions of Ghana to investigate diversity within yeast varieties involved in the spontaneous fermentation of Dagarti *pito*. Samples of dry yeast were obtained from commercial Dagarti *pito* producers from Tamale and Nyankpala (Northern Region); Ayigya- Kumasi and Monaco-Kumasi (Ashanti Region); Accra (Greater- Accra Region); Cape Coast (Central Region); Takoradi (Western Region); Sunyani (Brong Ahafo Region); Ho (Volta Region) and Suhum (Eastern Region). For purposes of comparison, dry yeast was also sampled from three *dolo* production sites in Ouagadougou, Burkina Faso. Yeast populations ranged between 10^6 and 10^8 cfu $g^{-1.}$ Twenty-five yeast isolates from each site were characterized phenotypically by colony and cell morphology as well as carbohydrate assimilation profiling, using the API ID 32 C Kit (Biomerieux SA, Marcy L'Etoile, France). Ninety-nine percent (247) of the isolates showed colony and cell morphologies typical of *S. cerevisiae*. Of these, 72 % (179) had fifty-three carbohydrate assimilation profiles similar to *S. cerevisiae* (according to Vaughan-Martini and Martini, 1998) and were subsequently identified as such while 28 % (68) which had four carbohydrate assimilation profiles atypical of *S. cerevisiae* or any other member of the *sensu stricto* complex could not be identified in API galleries. Two isolates (1%) which had colony and cell morphologies atypical of *S. cerevisiae*, and a broad-spectrum assimilation profile, were identified as *Candida kefyr*. Genotyping of five randomly selected isolates from each site was carried out

using the Polymerase Chain Reaction (PCR) to amplify the region spanning the two intergenic transcribed spacers (ITS) and the 5.8S ribosomal gene (ITS1-5.8S rDNA-ITS2), followed by restriction analysis (ITS-PCR+RFLP) of the product, as well as Pulsed Field Gel Electrophoresis (PFGE). The genetic analyses indicated that all of them belonged to *S. cerevisiae*, notwithstanding the phenotypic differences. The mitochondrial cytochrome-*c* oxidase II gene (*COX 2*) of four isolates representing the four chromosome profile groupings that emerged after PFGE, were then sequenced to confirm their close relatedness to *S. cerevisiae*, particularly type strain CBS1171. Two isolates randomly selected from each of the ten production sites, (one with a broad carbohydrate assimilation spectrum and the other with a narrow carbohydrate assimilation spectrum) and assessed for technological properties showed different patterns of growth and flocculation without much change in pH during fermentation, and most of them produced *pito* having sensory attributes which compared favorably with commercially produced *pito*. *Pito* produced with each of ten out of the twenty yeast strains from Ghana used for the earlier investigations and three from Burkina Faso was analyzed by headspace, for its aroma constituents. All ten Ghanaian isolates could form aromatic compounds representing the alcohols, esters, and ketones which are among reported typical flavor compounds of conventional beer. This study has demonstrated diversity within *S. cerevisiae* strains involved in fermentation of *pito* wort. These strains possess desirable technological properties, including sufficient growth during fermentation and efficient hydrolysis of sugars for biomass

enhancement. They also demonstrated fermentation activities, particularly, ethanol production, formation of aroma compounds and metabolites, which impart appropriate sensory attributes to *pito*.

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background and Justification

Fermented products play very significant roles in the diet of the peoples of West Africa. It has been established that nearly two-thirds of the staple food intake of Ghanaians, (for example, *kenkey, banku,* and *tuo zafi),* are some form of fermented product (Halm *et al.*, 1996). In southern Ghana, about 40% of these foods are maize-, and cassava-based. In the northern regions, however, several fermented foods and beverages are produced from sorghum (*Sorghum vulgare*) also known as Guinea corn, and millet (*Pennisetum typhoideum*). These include *tuo zafi, foro-foro, porha beer*, and *pito.* A preliminary survey conducted by the Food and Nutrition Security Unit of the University for Development Studies, Tamale on *Pito* Brewing and Consumption in Tamale Municipality in 2001 rated *pito* as the most popular and highly consumed alcoholic beverage produced by fermenting sorghum wort (unpublished information).

Pito is brewed and consumed all over the three northern regions and in the northern parts of the Brong-Ahafo region. Conservative estimates by the Revenue Task Force of the Tamale Municipal Assembly (TMA) in 2001 gave a total of sixty (60) *pito* bars in Tamale alone, each with an average daily patronage of 100 persons and sales of 200 litre-sized pots of *pito* (i.e., 12, 000 litres total day). *Pito* is also

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

The cultural heritage of virtually every civilization includes one or more fermented foods made by the souring action of microbes. *Leban* (Egypt and Syria), *taettemjolk* (Scandinavia), *matzoon* (Armenia), *dahi* (India), *piner* (Lapland), *wara* and *waranski* (Sudan and Niger), *yakult* (Japan), *kefir* (Bulgaria) and the blue-veined cheese produced by the fungus *Penicillium roqueforti* (France), are some well-known examples. Men and women have, therefore, through succeeding generations, found ways to use a mix of microbes and traditional domestic skills to make new nutritional foods that tasted better and kept longer. While the western world can afford to enrich its foods with synthetic vitamins, the developing world must rely upon biological enrichment for its vitamins and essential amino acids. The affluent western world cans, chills and freezes much of its food but the developing world must rely upon fermentation and solar dehydration to preserve and process its foods at costs within the means of the average consumer. All consumers have a considerable portion of their nutritional needs met through fermented foods and beverages (Steinkraus, 1996).

2.2 Survey of fermented products over the world

Selected fermented products from Asia, Europe, Americas/Caribbean, and Africa are presented in Tables 2.1 (a) and 2.1 (b).

2.3 The role of fermentation

Fermentation is generally regarded as one of the economical methods of producing and preserving foods for human consumption (Pederson, 1971). Fermentation has been found to play five major roles:

I. *Enrichment of diet through development of a diversity of flavors, aromas, and textures in food substances.*

Fermented foods have been described as food substrates that are invaded or overgrown by edible microorganisms whose enzymes; particularly amylases, proteases and lipases hydrolyze the polysaccharides, proteins, and lipids to non-toxic products with flavors, aromas and textures pleasant and attractive to human consumers as well as ensuring their consistency (Steinkraus, 1996).

II. *Preservation of substantial amounts of food through lactic acid, acetic acid, alcoholic, and alkaline fermentations.*

According to Odunfa (1985a), fermented products have the advantage of prolonged shelf life due to organic acids. For example, *ogi*, an infant weaning food, can be kept for 10 days and over by changing the water every 48 hours. Lactic acid, acetic acid, and other acids formed during the fermentation process lower the pH, thus inhibiting the growth of spoilage organisms.

III. *Enrichment of food substrates biologically with protein, essential amino acids, essential fatty acids, and vitamins.*

Many workers (e.g., Fetuga *et al*., 1973; Eka, 1980; Odunfa, 1985a; Sanni, 1988; Ouoba *et al*., 2003) have reported the enhanced nutritional value of fermented products as compared to the unfermented substrates.

IV. *Detoxification during food fermentation processing.*

It has been reported that fermentation decreases or eliminates completely toxic components in some products such as fermentation of grated cassava pulp to produce *gari* and *lafun* (Ikediobi and Onyike, 1983; Oyewole and Odunfa, 1988; Oyewole and Odunfa, 1990), and fermentation of castor oil-bean seed for *ogiri* production (Odunfa, 1985b; Sanni and Ogbonna, 1991). According to Sharma and Kapoor (1996), fermentation may also lead to the degradation and destruction of undesirable factors present in raw foods such as phytates, tannins and polyphenols. Some Lactic Acid Bacteria (LAB) and yeast strains associated with fermented foods are capable of degrading antinutritional factors, such as phytic acid (whose chelating properties may significantly reduce the bioavailability of minerals such as calcium, iron, magnesium and zinc) and phenolic compounds (from millet and sorghum) (Holzapfel, 2002).

Other workers (e.g., Sanni, 1993; Padmaja, 1995; Iwuoha and Eke, 1996; Addo *et al*., 1996; Amoa-Awua *et al*., 1997; Svanberg and Lorri, 1997; Odunfa and Oyewole, 1998; Onilude *et al*., 1999; Sindhu and Ketarpani, 2001) have reported of

beneficial effects of fermented foods and beverages in developing countries such as reduced loss of raw materials, reduced cooking time, improvement in protein quality and carbohydrate digestibility, improved bio-availability of micronutrients, and elimination of toxic and anti-nutritional factors such as cyanogenic glycosides (e.g. linamarin and lotaustralin in cassava).

V. *Probiosis*

Many other workers (Mensah *et al*., 1990, 1991; Nout, 1991; Mensah, 1997; Kimmons *et al*., 1999 and Lei and Jakobsen, 2004) have attributed the reduction in the severity, duration and morbidity of diarrhoea in developing countries to the probiotic effects and the reduced level of pathogenic bacteria seen in fermented foods and beverages.

2.4 African indigenous fermented foods and beverages

Fermented foods and beverages play a predominant role in the diet of African people (Steinkraus, 1996). Workers such as Van der Walt (1956), Haggblade and Holzapfel (1989), Ashenafi (1990), Dirar (1993), and Mwesigye and Okurut (1995) have documented several traditional fermented products in different African countries which include non-alcoholic and alcoholic beverages, breads, pancakes, porridges, cheeses and milks. According to Sanni (1993), these foods are most often produced at the household level or at small industrial scale and are consequently often of varying quality and stability. Cereals, legumes, and root

tubers are the major raw materials in Africa, but other raw materials such as milk, fish, and meat may also be fermented (Steinkraus, 1996).

2.4.1 Fermented non-alcoholic foods and beverages

These are prepared from cassava, a root tuber, one (or combinations) of such cereals as sorghum, millet, and maize; vegetables proteins, and a few other raw materials.

Oyewole and Odunfa (1988) reported that *gari*, a granular starchy food made from cassava (*Mannihot utilissima*, *M. esculenta*) by fermenting the grated pulp, followed by semidextrinizing, drying, and grading, is a major staple diet in Nigeria. According to Akinrele *et al*. (1965), about 10 million tons of *gari* are produced per annum in the southern part of Nigeria alone, while substantial quantities are also produced along the other coastal regions of West Africa. Other cassava-based fermented foods are *lafun* (a cassava flour)(Oyewole and Odunfa, 1988), *fufu* (Abioye, 1981) from Nigeria; *agbelima* (cassava dough) from Ghana (Amoa-Awua *et al*., 1997); *chikawangue* from Zaire (Odunfa, 1988) and *cingwada* from East and Central Africa (Odunfa, 1988).

Examples of cereal-based non-alcoholic fermented foods include *ogi*, a fermented porridge popular in various parts of West Africa (Akinrele, 1970); *koko* and *kenkey* from Ghana (Andah and Muller, 1973); *mahewu (magou)* from South Africa (Hesseltine, 1979); *tobwa* from Zimbabwe (Gadaga *et al*., 1999); *uji* from East

Africa (Mbugua, 1981); *kisra* from the Sudan (Dirar, 1993); *enjera* from Ethiopia (Ashenafi, 1994); and *mawe* from Benin and Togo (Hounhouigan *et al*., 1993).

Vegetable proteins from the seeds of indigenous leguminous plants are fermented and used mainly as soup condiments and additives for flavor and aroma, for example, *iru* from West and Central Africa (Odunfa, 1981); *owoh* from Mid-western Nigeria (Sanni and Ogbonna, 1991); *ogiri* from Southern Nigeria (Odunfa 1985a); *ugba* from Eastern Nigeria (Odunfa and Oyeyiola, 1988) and Sierra Leone (Obeta, 1983); *kawal* from Sudan (Dirar, 1984); *soumbala* from Burkina Faso (Ouoba *et al*., 2003) and *kantong* from Ghana (Kpikpi, 2006).

2.4.2 Fermented milks

The most abundant fermented animal product in Africa is milk. According to Odunfa and Oyewole (1998), fermented milk is mostly used in East Africa and in the Kenyan highlands where up to 53% of the milk produced is reportedly consumed as fermented milk. Abdelgadir *et al*. (1998) and Gadaga *et al*. (2000) have reported that fermented milk also constitutes an important part of the traditional diet in Sudan and Zimbabwe. These products, which take the form of sour milk, yogurt, traditional butter or cheese, include *mukaka wakakora*, or *amasi* and *hodzeko* from Zimbabwe (Gadaga *et al*., 1999); *ergo, ititu, kibe*, and *ayib* from Ethiopia (Gonfa *et al*., 2001); the Sudanese *rob, gariss, biruni* and *mish*

(Abdelgadir *et al*., 1998); *nono* from Nigeria (Okagbue and Bankole, 1992); and *mbanik* from the Senegal (Gnigue *et al.*, 1991).

2.4.3 Alcoholic beverages

Alcoholic beverages are fermented products in which sugars are the principal fermentable carbohydrates and which, consequently, have ethanol as a major component.

According to Sanni *et al*. (1999), various kinds of traditional alcoholic beverages are produced in Nigeria and other West African countries from many types of agricultural substrates. These beverages can be categorized into: (i) those produced from fermented sugary sap or fruit juice, and (ii) those produced from malted cereal grains.

2.4.3.1 Alcoholic beverages produced from sugary sap or fruit juices

Fermented sugary sap (palm wine) from the oil palm tree (*Elaeis guineensis*) and the raffia palm (*Raphia hookeri*) is called *emu* in Nigeria (Okafor, 1972). This drink is milky white, effervescent with sweet taste. *Agadagidi* is reported by Sanni and Oso (1988) to be another alcoholic beverage from South-western Nigeria and Cameroon, produced from ripe plantain pulp.

2.4.3.2 Cereal-based alcoholic beverages

Cereal-based alcoholic beverages have heavy consistency because of suspended undigested starch granules, and the microorganisms responsible for the fermentations (Okafor, 1990).

Maize-based alcoholic beverages include *sekete* from South-western Nigeria (Sanni, 1988), *malawa* beer from Uganda (Odunfa and Oyewole, 1998), and *busaa* from Kenya (Nout, 1980). Other alcoholic beverages produced from mixtures of malted sorghum and maize (and/or millet), include *kaffir* beer of the Bantus of South Africa (Novellie, 1963) and *doro/uthwala* and *chikokivana* from Zimbabwe (Gadaga *et al*., 1999).

Sorghum-based alcoholic beverages are by far the most popular in Africa. Known under a variety of names, they include *burukutu* and *otika* from Nigeria and Ghana (Faparusi *et al*., 1973; Sefa-Dedeh and Asante, 1988), *bili-bili* from Northern Cameroon (Nso *et al.*, 2003), *dolo* from Burkina Faso (Konlani *et al*., 1996), and *pito* from Nigeria, Togo and Ghana (Ekundayo, 1969; Sefa-Dedeh and Asante, 1988; Bansah, 1990). These beverages, apart from serving as inebriating drinks, are also important in fulfilling social obligations such as marriage, naming and burial ceremonies (Sanni and Lonner, 1993).

2.5 Microbiology of African indigenous fermented foods and beverages

Microorganisms characteristic of indigenous fermented foods and beverages are generally edible. According to Steinkraus (1996), those with unusual ability to

produce amylolytic, proteolytic, lipolytic, pectinolytic or other enzymes, vitamins, essential amino acids, essential fatty acids, antibiotics, organic acids, peptides, proteins, fats, complex polysaccharides, compounds with unusual or desirable flavors, or flavor-enhancing compounds are of potential value to the food industry. Lactic acid bacteria and yeasts have been reported by several workers including Nout (1991) and Halm *et al.* (1993), to be the predominant microorganisms in most African indigenous fermented foods. Also, Gobbetti *et al*. (1994) indicate that stable co-metabolism between LAB and yeasts is common in many foods, enabling the utilization of substances that are otherwise nonfermentable (for example starch) and thus increasing the microbial adaptability to complex food systems. Nout (1991) has also suggested that the proliferation of yeasts in foods is favored by the acidic environment created by LAB while the growth of bacteria is stimulated by the presence of yeasts, which may provide growth factors, such as, vitamins and soluble nitrogen compounds. It has been asserted by Akinrele (1970) and Halm *et al*. (1993) that the association of LAB and yeasts during fermentation may also contribute metabolites, which could impart taste and flavor to foods while Nout *et al*. (1989) and Mensah *et al*. (1991) have suggested that the production of lactic acid (in *gruel*) during fermentation may promote or improve the microbiological safety and stability of the products.

According to Jespersen (2003), *Saccharomyces cerevisiae* is the yeast species most often reported in African indigenous fermented foods and beverages, having been isolated from fermented non-alcoholic starchy foods, alcoholic beverages and

fermented milk. Most of these products are fermented spontaneously or by 'back-slopping', that is, inoculation with a part of a previous fermentation, as in the fermentation of cassava for *agbelima* production (Amoa-Awua *et al*., 1997) and fermentation of sorghum wort for *pito* production (Sefa-Dedeh and Asante, 1988; Bansah, 1990). For spontaneous fermentations, yeasts have been reported by Jespersen *et al*. (1994) to originate from raw materials and process equipment.

In the fermentation of maize dough for *kenkey* production in Ghana, for instance, *S. cerevisiae* were found by Jespersen *et al*. (1994) and Halm *et al*. (1993) to be predominating after 24 - 48h among other species like *Candida, Trichosporon, Kluyveromyces*, and *Debaryomyces*. Hounhouigan *et al*. (1999) have also reported *S. cerevisiae* and *Lactobacillus* spp. as the dominant microorganisms in the production of *mawe*, a porridge made from dehulled and partially germinated white maize in Benin. *Saccharomyces cerevisiae* has also been reported by Sanni (1993) to be involved in the fermentation of *ogi*, another acid-fermented food based on maize, sorghum or millet that is used as a traditional weaning food all over Africa, but is also eaten by adults. The predominant microorganisms involved in production of *kisra* have been reported by Steinkraus (1996) to be *S. cerevisiae, Lactobacillus* spp., and *Acetobacter* spp.

The involvement of *S. cerevisiae* in fermented milk products has been reported in the production of *hodzeko, mukaka wakakora*, or *amasi* from Zimbabwe by Gadaga *et al*. (2000), *rob* from Sudan by Abdelgadir *et al.* (2001), *nono* from Nigeria by Okagbue and Bankole (1992) and *mbanik* from Senegal by Gnigue *et al.* (1991).

Alcoholic beverages in which *S. cerevisiae* is associated with the fermentation process include *busaa* from Kenya (Steinkraus, 1996); *malawa* beer from Uganda (Odunfa and Oyewole, 1998), *munkoyo* from Zambia (Zulu *et al*., 1997); palm wine from Nigeria, Ghana and several other coastal countries of West Africa, *agadagidi* from Nigeria and Cameroon, and *pito* from Nigeria, Togo and Ghana (Ekundayo, 1969; Sefa-Dedeh and Asante, 1988).

2.6 *Pito*

Pito is a sorghum-based alcoholic beverage common to the peoples of Nigeria, Togo and Ghana (Ekundayo, 1969; Sefa-Dedeh and Asante, 1988). It is golden-yellow to dark-brown in color with taste varying from slightly sweet to sour and contains lactic acid, sugars, amino acids, 2 - 3% alcohol (v/v), as well as some vitamins and proteins (Ekundayo, 1969; Bansah, 1990). Compared with European beers, *pito* is heavier and darker, but less bitter, lighter in color and thinner in consistency than European stout beer (Ekundayo, 1969).

In Nigeria, *pito* is produced in the western and northern states, and serves as the main source of income for many women who learn the art during adolescence and subsequently brew it once or twice a week. It is usually consumed as a nutritious beverage following a light meal; during beer parties on market days and on Sundays, at wedding parties, cultural festivals, and collective work gatherings. It is also served as refreshment during leisure hours (Ekundayo, 1969).

In Ghana, *pito* brewing is traditionally associated with women in the northern parts of the country, but migration has led to its production throughout the whole country (Sefa-Dedeh and Asante, 1988). Therefore, the production of *pito* does not only provide psychological and social enjoyment, but also has important implications for the food system and economy of the country. Four types of *pito* are produced in Ghana – "Nandom", "Konkomba", "Togo", and "Dagarti". The peculiar characteristics of each type lies in the methods of wort extraction and alcoholic fermentation (Sefa-Dedeh *et al.*, 1999). In "Togo" and "Konkomba" *pito* production, the milled malt is mixed with water and left overnight to form a mash, while in the other types ("Dagarti" and "Nandom"), the mash is mixed with slime extracted from crushed okro stems (*Albelmucus esculentum* L. Moench) or a decoction of pulped bark of the *yolga* plant (*Grevia bicolor*) to facilitate sedimentation (Demuyakor and Ohta, 1991; Mary Benyima [Personal Com.]). During the alcoholic fermentation of "Konkomba" and "Togo *pito*", yeast from previously-brewed *pito* trapped in the interstices of a traditional woven belt which consists of flax or hennep are used for the inoculation of the wort, whereas a portion of a previous fermentation or dried yeast (*dambeli*) derived from the top foaming part is usually added for the alcoholic fermentation of wort in "Dagarti" and "Nandom *pito*" production (Sefa-Dedeh and Asante, 1988; Bansah, 1990; Demuyakor and Ohta, 1991).

2.7 Steps in *pito* production

Pito brewing, like any conventional beer production, basically involves malting, mashing, and fermentation.

Malting essentially comprises steeping, germinating, and limiting cereal growth when enzymes have been produced for the degradation of starch and proteins in the cereal grain. Indeed, the malting quality of sorghum depends upon physical and biochemical activities such as temperature and time of storage of the grains, steeping period, germination, kilning temperature (Novellie, 1962; Pathirana *et al*., 1983; Owuama and Asheno, 1994) and sorghum cultivars (Subramanian *et al*., 1995).

Time and temperature of storage influence the percentage soluble amylases in sorghum grains. For example, sorghum grains stored at 12 to 23˚C for 2 to 3 years give higher levels of soluble amylase (between 57 and 73%) while newly harvested grains give about 25 percent (Novellie, 1962). Lowering the storage temperature to 7˚C reduces the level of soluble amylase in the grains to about 31% after 3 years (Novellie, 1962). Sorghum for *pito* production is often purchased from the open market. There is therefore a high risk of using inappropriately stored sorghum grains, resulting in low quality products.

Steeping, which involves the soaking of grains in water until an acceptable moisture level is reached, allows for the removal of some pigments, microorganisms and bitter substances from selected grains. The methods of steeping (that is, with and without change of water) have virtually no effect on the

malting of sorghum (Novellie, 1962). During steeping, certain physical and biochemical changes occur, such as the swelling of the grains and the degradation of degradable carbohydrates (Novellie, 1962). In traditional *pito* production, the sorghum grains are manually sorted and sparingly cleaned before steeping in buckets or big bowls, which may or may not be covered. There is therefore a strong likelihood of finding foreign materials such as stones, strands of hair, rodent and bird droppings and other debris in the grains during steeping. In areas without potable (clean) water, the water for steeping is likely to be derived from ponds, rivers and wells, which may have doubtful hygienic quality.

Germination involves the outgrowth of the plumule and the radicle of the seedling until adequate enzymes have been produced for the malt, but before the exhaustion of the seed nutrients. However, germinating sorghum grains heavily infected with mould produce malts with slightly higher amylase activity resulting in off-flavour as well as mycotoxins production (Kumar *et al*., 1992). Seed mycoflora of sorghum species include *Aspergillus flavus, Curvularia lunata, Cladosporium cladosporoides, Fusarium moniliforme, Rhizopus* spp., *Altenaria* spp., *Penicillium* spp., *Dreschlera* spp., and *Neuropora* spp. (Faparusi *et al*., 1973; Kumar *et al*., 1992; Boboye and Adetuyi, 1994). Steeped sorghum grains for *pito* production are spread out on washed cement floors and covered with jute sacks for germination in rooms where there is little control over germination conditions like temperature, humidity, aeration, etc. Hurriedly washed floors and unclean jute sacks are potential sources of contaminants such as moulds as well as spoilage bacteria. Lack

of control over germination conditions could also lead to under- or over-germination of grains, which may affect the malt quality.

Prior to malting, only a small proportion of β-amylase of cereals such as wheat, rye, barley, and sorghum is soluble (Novellie, 1960; Owuama and Okafor, 1990). Malting yields higher proportions of hydrolytic enzymes such as α- and β-amylases, which may be either completely soluble or largely insoluble depending on the variety of sorghum (Novellie, 1960; Jayatissa *et al*., 1980; Demuyakor and Ohta, 1992). During malting (germination) and subsequent processing, amylases from the germinated sorghum grains hydrolyze the starches to fermentable sugars, principally maltose and glucose (Novellie, 1960; Faparusi, 1970). The moulds on the grains (e.g., *Rhizopus oryzae, Aspergillus flavus, Penicillium funiculosum* and *P. citrinum*) may also contribute amylases (Platt and Webb, 1946). Malting causes a decrease in the density of caryopsis in sorghum grain (Beta *et al*., 1995), lowers the amount of lysine from 0.25% in unmalted sorghum to 0.18% in malted sorghum (Okoh *et al*., 1989), and reduces the milling energy (Swanston *et al*., 1994). The milling energy shows significant positive correlation with the amount of soluble nitrogen in the extract but correlates negatively with diastatic power and sedimentation rate (Lasekan, 1993). Fine milling (that is, 0.2 mm particle size) of malt increases hot water extract, diastatic power and sugar contents (Lasekan *et al*., 1995). Storing malt for any period of time has virtually no effect on the soluble amylase content (Novellie, 1960).

Kilning, like sun drying, which involves drying of the green malt until the rootlets become friable or brittle, contributes to color development (Briggs *et al*., 1981). Green malt is sun-dried during *pito* production, thus making the process uncontrolled and dependent on environmental conditions and the discretion of producers. Color development will therefore vary from producer to producer and, period to period.

Mashing in conventional brewing is basically by two methods; decoction and infusion processes (Hough *et al*., 1971). Mashing involves: (i) dissolving substances, which are directly soluble in water; (ii) enzymic hydrolysis, followed by dissolution of a series of substances important for the type and character of beer and (iii) separation of the dissolved substances.

Enzymes involved in the hydrolysis of substances include amylases, proteases, peptidases, transglucosidases and phosphorylases. Factors such as temperature, pH, time, and concentration of the wort regulate the activities of these enzymes (Hoyrup, 1964). During mashing, rapid degradation of solubilized starch and proteins and to a lesser extent other higher molecular weight substances occurs. Mashing extracts contain about 80% of the dry matter from the malt, while cold water extracts contain about 15% (Wainwright, 1971).

Protein seems to play a minor role in determining the quality of sorghum malt as high protein content in sorghum malt causes no brewing problems (Novellie, 1962). This is attributed to the apparent degradation of most of the high molecular weight

proteins into simpler compounds during mashing and the removal of coagulated protein sediment, which results from wort boiling (Novellie, 1962).

2.7.1 Microbiology of *pito* fermentation

Pito production is by spontaneous and uncontrolled mixed fermentation involving lactic acid bacteria and yeasts. Bacteria of the genus *Lactobacillus* and *Weisella* spp. are the major contributors to the acidity of *pito* during the initial souring (acidification) stage (Steinkraus, 1996; Lingani-Sawadogo *et al*., 2010). Most of the acid produced is lactic acid with only traces of acetic and formic acid being present. Accumulation of acetic acid gives *pito* a vinegary flavor, which is less acceptable (Steinkraus, 1996).

A study by van der Aa Kühle *et al*. (2001) reported the almost exclusive occurrence of *Saccharomyces cerevisiae* strains (99%) in yeast associated with production of Dagarti *pito* and *dolo* from northern Ghana and neighboring Burkina Faso, respectively; the remaining 1% being *Candida kefyr*. This is in contrast to earlier studies which reported the presence of small numbers of a few other yeast genera (Sefa-Dedeh and Asante, 1988; Bansah, 1990; Demuyakor and Ohta, 1991).

These reports, however, cover studies carried out in just one or two regions of Ghana. Characterization of *pito* yeast from all the regions of Ghana seems not to be reported and no link seems to be established between predominant yeast and *pito* quality.

2.8 Techniques for characterization and identification of yeast

Molecular techniques have become valuable in identifying and characterizing yeasts (Kurtzmann and Robnett, 1998).

Hayford and Jespersen (1999) used Pulsed-Field-Gel-Electrophoresis (PFGE) to distinguish between isolates from fermented maize dough having assimilation profiles typical of *S. cerevisiae*, by showing that nearly all of them displayed individual chromosome profiles. They further separated a few that could not be separated during PFGE, by PCR amplification using primers against the 5´-termini of the delta elements flanking the Ty1 retrotransposon. Again, van der Aä Kühle *et al*. (2001) employed PFGE and sequencing of the D1/D2 domain of the large subunit 26S rDNA to distinguish to strain level isolates from fermented sorghum beer (*pito* and *dolo* from northern Ghana and Burkina Faso respectively) showing phenotypic characteristics atypical of *S. cerevisiae* type strain CBS 1171 (Vaughan-Martini and Martini, 1998) but yet qualified to be considered as such according to Barnett *et al*. (2000). Chromosome length polymorphism determined by PFGE and similar techniques, have also been successfully used to differentiate between strains of *Saccharomyces* spp. within both wine and brewing yeast, brewing contaminants and baker's yeast by Bidenne *et al*. (1992), Vezinhet *et al*. (1992), Casey *et al*. (1990) and Jespersen *et al*. (2000).

Amplification by PCR of the region spanning the two intergenic transcribed spacers (ITS) and the 5.8S ribosomal gene (ITS1-5.8SrDNA-ITS2), followed by restriction analysis has also been employed in differentiating several yeast species including

Saccharomyces spp. (Guillamon *et al*., 1998; Esteve-Zarzoso *et al.*, 1999). Baleiras Couto *et al*. (1996) also found this method very useful in subspecies typing of *S. cerevisiae.*

Sequence analysis, which indicates the degree of nuclear DNA relatedness and genetic crosses between organisms, has become a preferred basis for establishing pairs of sibling species, in contrast to conspecific strains (Daniel and Meyer, 2003). Belloch *et al*. (2000) recently reported the usefulness of sequencing of the mitochondrial gene *COX 2*, encoding the cytochrome-*c* oxidase subunit II, in examining relationships among species of *Kluyveromyces.* Also, van der Aa Kühle and Jespersen (2003) have used this method in establishing that *S. boulardii* is closely related to *Saccharomyces cerevisiae*. The use of PFGE, ITS-PCR + RFLP and sequencing in the present study to characterize the yeast isolates from the various production sites would therefore lend credence to the identification of the isolates and thus give a clear picture of the yeast species involved in the alcoholic fermentation of sorghum wort to produce Dagarti *pito* in Ghana.

2.9 The potential of *Saccharomyces cerevisiae* as a starter culture in the small-scale industrial production of indigenous fermented foods in Africa

A starter culture may be defined as a preparation or material containing large numbers of viable microorganisms, which may be added to accelerate a fermentation process. Being adapted to the substrate, a typical starter culture facilitates improved control of a fermentation process and predictability of its

product (Holzapfel, 1997). In addition, starter cultures facilitate control over the initial phase of a fermentation process as exemplified in 'back-slopping' which shortens the process thereby reducing the risk of fermentation failure (Holzapfel, 2002). The development of starter cultures is one of the prerequisites for the establishment of small-scale industrial production of fermented foods in Africa (Sanni, 1993).

Kaffir (Bantu) beer from South Africa and *doro* from Zimbabwe and Botswana are probably the only indigenous fermented beverages produced by modern industrial processes (Sanni, 1993; Pattison *et al*., 1998; Gadaga *et al*., 1999). *Saccharomyces cerevisiae* is used as starter culture for both *kaffir* beer and *doro* (Pattison *et al*., 1998; Gadaga *et al*., 1999). Commercial starter cultures of *S. cerevisiae* have also been reportedly used by Gadaga *et al*. (1999) in the production of *chikokivana* in Zimbabwe.

Saccharomyces cerevisiae strains isolated from African indigenous fermented products have properties different from the *S. cerevisiae* type strain CBS 1171 (Hayford and Jespersen, 1999; van der Aa Kühle *et al*., 2001). For example, even though all the isolates from fermented maize dough had assimilation profiles typical of *S. cerevisiae*, and had chromosomal band patterns with size distribution typical of *S. cerevisiae*, there was evidence of chromosome length polymorphism among both isolates from different production sites and within the same batch of fermentation (Hayford and Jespersen, 1999). To be accepted as *S. cerevisiae*, an isolate must be able to assimilate glucose, sucrose, maltose, trehalose, raffinose and

ethanol (Vaughan-Martini and Martini 1998). Fifty-three percent of isolates from fermented sorghum beer (*pito/dolo*) produced in the northern part of Ghana and Burkina Faso by (van der Aa Kühle *et al*., 2001) were able to assimilate only glucose and maltose and thus were not consistent with the accepted description of the species of *S. cerevisiae* by these authors but qualified to be considered as such in accordance with the description given by Barnett *et al.* (2000). These isolates also exhibited pronounced chromosome length polymorphism under pulsed-field gel electrophoresis with each isolate having an individual chromosome profile. No correlation was seen between the clustering of the chromosome profiles and the assimilation pattern of the isolates. Sequence analysis of the D1/D2 domain of their large subunit (26S) rDNA revealed a deviation from the type strain of *S. cerevisiae* (CBS 1171) of three nucleotides, equivalent to 0.5% of the DNA (van der Aa Kühle *et al*., 2001). It is therefore imperative that starter cultures for indigenous fermented foods and beverages are isolated from the products they are supposed to be used for, and selected according to the technological properties required for each product (Jespersen, 2003).

Reported examples of situations where strains of *S. cerevisiae* have been isolated from indigenous fermented foods and beverages and thereafter successfully used as starter cultures are Ghanaian fermented maize dough for *kenkey* and *koko* production (Halm *et al.*, 1996; Annan *et al*., 2003a), Ghanaian fermented sorghum beer-*pito* (Sefa-Dedeh *et al*., 1999; Glover *et al*., 2005), Zambian *munkoyo* maize beverage (Zulu *et al*., 1997), and Nigerian maize – based *ogi* (Teniola and Odunfa,

2001). In the case of *pito*, however, these starter cultures are crude and unrefined, having been obtained as dried yeast from the top foaming part of fermenting *pito* (*dambeli*) in the "Dagarti" and "Nandom" types, or yeast from a previous fermentation trapped in the interstices of a traditional woven belt for the "Konkomba" and "Togo" varieties.

These have been applied by the traditional producers without any standardization and quality system considerations. It is imperative to develop *S. cerevisiae* strains isolated from *pito* into starter cultures based on standard scientific procedures, which will ensure microbiological safety and consistent organoleptic quality of the final product. Owing to the nation-wide production and consumption of *pito* in Ghana, the selection of strains should reflect a wide geographical setting to ensure that developed starter cultures can be used to produce *pito* of same quality anywhere in Ghana. Some LAB and yeast strains associated with fermented foods are capable of degrading antinutritional factors such as phytic acid and phenolic compounds. Incorporation of these organisms into starter cultures may, therefore, serve to upgrade the nutritional value of foods. Furthermore, selected strains may enhance the general benefits of spontaneous fermentation such as improved protein digestibility and micronutrient bioavailability, and contribute more specifically to biological enrichment through the biosynthesis of vitamins and essential amino acids (Holzapfel, 2002).

2.9.1 Handling, maintenance, and distribution of starter cultures for small-scale fermentations

Perhaps the oldest traditions in the preparation, handling and distribution of starter cultures are to be found in the different regions of Asia (Lee and Fujio, 1999). A typical example is the *ragi*-type starter cultures, mixed-culture dough inocula which have been used for centuries in the production of a variety of sweet and sour alcoholic beverages and pastes (Steinkraus, 1997; Tamang, 1998). Even though relatively little information is available on starter culture traditions in sub-Saharan Africa, the use of 'back-slopping' approaches for inoculation are widespread in the region (Holzapfel, 2002).

An example of a preserved starter is the inoculation belt for "Konkomba" and "Togo *pito*" fermentation, used in Ghana and Togo (Sefa-Dedeh and Asante, 1988; Bansah, 1990; Demuyakor and Ohta, 1991). The inert surface of the belt or woven rope, which consists of flax or hennep, facilitates the preservation of essential microorganisms (yeasts) during drying and storage (Holzapfel, 2002). The top foaming part of a fermenting batch of "Dagarti" or "Nandom *pito*" is dried to give a product called *dambeli* (dried yeast) another form of starter usually added for the alcoholic fermentation of wort in production of these two types of *pito* in Ghana (Sefa-Dedeh and Asante, 1988; Bansah, 1990; Demuyakor and Ohta, 1991). According to Holzapfel (2002), sun drying may destroy some microorganisms and thereby reduce viable numbers, while slow and insufficient air drying during the rainy season may result in contamination and poor quality starters. So far, the use

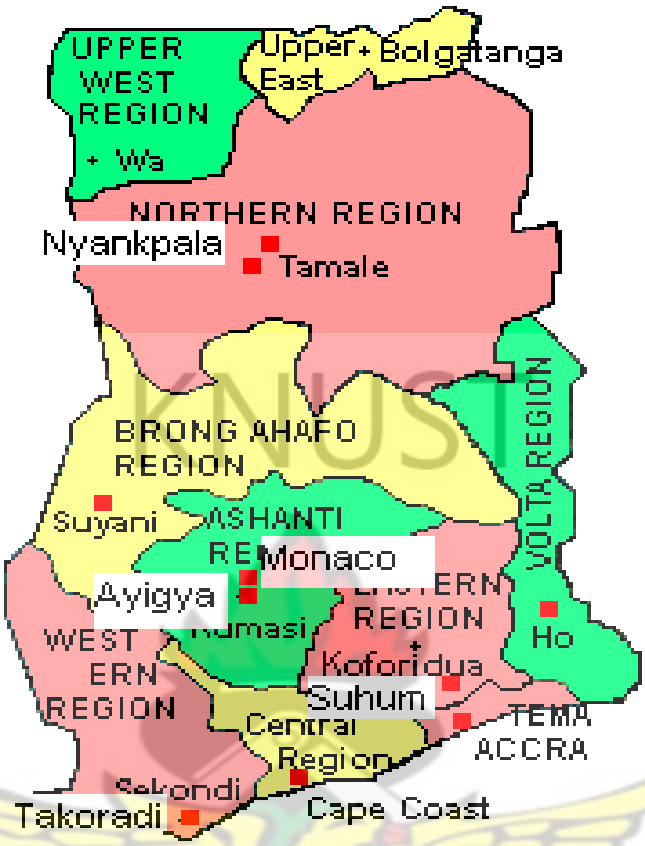
of sun-dried *pito* yeast has proven effective. Furthermore, the shelf life of dehydrated starters may be enhanced by storage in an airtight container. Bakers yeast is commonly used in the fermentation of sorghum and other cereal beers in Africa (Holzapfel, 1989).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area and origin of isolates

The study covered ten (10) production sites located in nine towns within eight administrative regions of Ghana (Plate 3.1).



MAP 3.1: Political map of Ghana showing its ten regions, including the eight from which ten sampling sites were selected.

The locations comprised Tamale and Nyankpala (Northern Region), Kumasi (Ashanti Region), Accra (Greater- Accra Region), Cape Coast (Central Region), Takoradi (Western Region), Sunyani (Brong Ahafo Region), Ho (Volta Region), and Suhum (Eastern Region).

One-on-one interviews were conducted with randomly identified commercial producers of "Dagarti *pito*" in the various locations on their origin, educational background, how they got into *pito* production and most importantly, production procedures. After a comparison of the production processes described by the

various producers, a composite flow diagram was drawn for "Dagarti *pito*" production (Fig.4.1).

Samples of dry yeast were obtained from each producer, Tamale and Nyankpala samples denoted T and N; Ayigya- Kumasi and Monaco-Kumasi samples denoted A and M; Accra samples denoted AC; Cape Coast samples denoted CC; Takoradi samples denoted TK; Sunyani samples denoted SY; Ho samples denoted HO; and Suhum samples denoted SH. For purposes of comparison, dry yeast was also sampled from three *dolo* production sites in Ouagadougou, Burkina Faso (samples denoted S, Z, and G).

3.2 Isolation of yeast

For determination of yeast colony-forming units (cfu), one gram (dry weight basis) of each dry yeast sample was crushed aseptically in a mortar, suspended in sterile saline peptone water (0.1% bactopeptone [Oxoid, Hampshire, England], 0.8% NaCl [Merck, Darmstadt, Germany]), pH 5.6 and incubated at 30ºC for 90 min. From 10-fold serial dilutions in saline, 0.1ml portion was surface-spread onto Malt extract Yeast extract Glucose Peptone (MYGP) agar (3 g malt extract [Oxoid], 3 g yeast extract [Oxoid],

5 g bactopeptone [Oxoid], 10 g glucose [Merck] and 20 g bactoagar [Oxoid] in a liter of distilled water, at final pH of 5.6 ± 0.1), supplemented with 100 mg of chloramphenicol (Oxoid) and 50 mg of chlortetracycline hydrochloride (Sigma, St. Louis, MO, USA). The culture plates were incubated at 30ºC for 3 - 5 days and

colony-forming units were enumerated. Twenty-five colonies were randomly selected from plates with distinct colonies, recultivated in MYGP broth at 30ºC for 2 days and further purified on MYGP agar (without antibiotics).

3.3 Phenotyping of *pito* yeast isolates

Colony characteristics (size, color, elevation, shape, texture, margin, and surface type) were determined for all isolates. Phase contrast microscopy was employed to determine cell shape, size, type of budding, and cell aggregation. The ability of isolates to assimilate various carbon sources was assessed using the API ID 32 C Kit (Biomerieux SA, Marcy L'Etoile, France).

The ID 32 C is an identification system for yeasts using standardized and miniaturized assimilation tests with a specially adapted database. A complete list of those yeasts that it is possible to identify with this system is usually found in an Identification Table inserted into the package.

The ID 32 C strip consists of 32 cupules, each containing a dehydrated carbohydrate substrate. A semi-solid, chemically defined, minimal medium is inoculated with a suspension of the yeast organism to be tested. After 24-48 hours of incubation, growth in each cupule is detected by visual reading. Identification is obtained using identification software. The list of substrates and their abbreviations are : Sorbitol (SOR); D-Xylose (XYL); Ribose (RIB); Glycerol (GLY); Rhamnose (RHA); Palatinose (PLE); Erythritol (ERY); Melibiose (MEL); Glucuronate (GRT); Melezitose (MLZ); Gluconate (GNT); Levulinate (LVT); Glucose (GLU); Sorbose (SBE); Glucosamine (GLN); Esculin (ESC); Galactose (GAL); Actidione

(ACT); Sucrose (SAC); N-Acetyl-Glucosamine (NAG); DL-Lactate (LAT); L-Arabinose (ARA); Cellobiose (CEL); Raffinose (RAF); Maltose (MAL); Trehalose (TRE); 2-Keto-Gluconate (2KG); α-Methyl-D-Glucoside (MDG); Mannitol (MAN); Lactose (LAC); and Inositol (INO). A control designated (0) was added.

In using the Kit, the strip was removed from its packaging, the desiccant discarded and the lid placed on it. The code of the yeast strain to be tested was recorded on the elongated flap of the strip. An ampoule of 2 ml Suspension Medium (Demineralized water) was opened as directed by the manufacturer and one or several identical colonies of yeast culture added to it to make a suspension with turbidity equivalent to 2 McFarland (McFarland Standard). Approximately 250 µl (5 drops of pipette) of the suspension was transferred into an opened ampoule of 7 ml C Medium (5 g Ammonium sulphate; 0.31 g Monopotassium phosphate; 0.45 g Dipotassium phosphate; 0.92 g Disodium phosphate; 0.1 g Sodium chloride; 0.05 g Calcium chloride; 0.2 g Magnesium sulphate; 0.005 g Histidine; 0.02 g Tryptophan; 0.02 g Methionine; 0.5 g Agar; 1 ml Vitamin solution; 10 ml Trace elements; added Demineralized water to 1000 ml; pH 6.4-6.8) and homogenized. The strip was inoculated by distributing 135 µl of suspension into each cupule with a micropipette. The lid was placed on the strip and incubated at 30 °C for 24-48 hours. The strips were visually read by comparing each cupule to the control (0) and any cupule that was more turbid was recorded as positive. The reactions obtained were coded into a numerical profile based on the assigned values of 1, 2 or 4 for each of the three groups into which the tests have been put on the result

sheet. The values within each group were then added together. Identification was obtained using the APILAB identification software by manually entering the 10-digit numerical profile: the 4 digits of the upper row (GAL-MDG), followed by the 4 digits of the lower row (SOR-LVT); the 9th digit for coding tests MAN, LAC and INO; and the 10th digit for GLU, SBE and GLN. The ESC test was not coded as it is only read if requested by the computer program in case of low discrimination between two species. Strips were re-incubated for further 24 hours when computer results indicated low discrimination, unacceptable or doubtful profile or "identification not valid before 48 hours incubation".

3.4 Genotyping of *pito* yeast isolates

3.4.1 ITS-PCR

Fifty representative isolates (five randomly selected from each site) and a type strain of *Saccharomyces cerevisiae* (CBS 1171) were pre-grown in 5 ml MYGP broth at 30 ºC for 2 days and cells harvested by centrifuging 3 ml of culture at 14000 × g for 5 min (OLE DICH Instrument Makers Aps, Copenhagen, Denmark). The pellets were resuspended in 200 µl TE-buffer (10 mM Tris-HCl (Sigma), 1 mM EDTA (Sigma)), boiled in the water bath (Grant Instruments, Cambridge, England) at 100ºC for 5 min and stored at -20ºC. DNA amplification (in 50 µl volumes) comprised 5 µl of 10 X PCR Buffer (Amersham Pharmacia Biotech, Uppsala, Sweden), 8 µl of 1.25 mMl^{-1} dNTP (dATP, dCTP, dGTP, dTTP)(Amersham Pharmacia Biotech), 4 µl of 25 mM l^{-1} $MgCl_2$ (Merck), 0.5 µl of

50 pM μl^{-1} each of forward and reverse primers, 0.5 µl of 1% (v/v) formamide (Amersham Pharmacia Biotech), 0.25 µl(2.5U) Taq-DNA Polymerase (Amersham Pharmacia Biotech), 30.25 µl of sterile Milli-Q water and 1 µl of template.

The forward primer--Y-ITS1 (5´-TCC GTA GGT GAA CCT GCG G-3´) (DNA Technology, Copenhagen, Denmark) and the reverse primer--Y-ITS4 (5´-TCC TCC GCT TAT TGA TAT GC-3´) (DNA Technology, Copenhagen, Denmark) were used. The PCR reaction was performed in a thermocycler (Biometra TRIO^{48}-Thermoblock^{TM,} Biotron, Göttingen, Germany) with a heated lid (TRIO Heated Lid, Biometra). Reaction conditions were: initial denaturing at 95 ºC for 5 min, 35cycles at 30 seconds each of denaturing at 95 ºC, annealing at 56 ºC and extension at 72 ºC, final extension at 72 ºC for 7 min and cooling to 4ºC. Amplified fragments were separated by electrophoresis on the GIBCO BRL Horizontal Gel Electrophoresis Apparatus, Horizon11.14 (Life Technologies$_{TM}$, Gaithersburg, MD, USA) with a 2%(w/v) Seakem GTG agarose gel (BioWhittaker Molecular Applications (BMA), Rockland, ME, USA) in 0.5×TBE (0.45 M Tris-base (Sigma), 0.45 M Boric acid (Sigma), 10 mM EDTA (Sigma)), with 4 µl ethidium bromide (10 mg ml^{-1}) (Sigma) added and with 0.5 × TBE as running buffer. A 10 µl portion of PCR product with loading dye (Promega, Madison, WI USA) were loaded into wells with 9 µl of øX174 DNA/*Hae* III (New England BioLabs Inc., Beverly, MA, USA) as size marker. The electrophoresis conditions generated by the daela PP3-89 power supply unit (Daela Electronik ApS, Copenhagen, Denmark) were 80 V and 40 mA for 60 min.

3.4.2 RFLP Analysis

Fifteen microlitres (µl) of PCR product was digested with 0.5 µl (5U) of *Hae* III enzyme

(New England BioLabs Inc.) in 2 µl NE Buffer 2 (New England BioLabs Inc.) and 7.5 µl sterile Milli-Q water and incubated overnight (16 hours) at 37 ºC. Restriction fragments were analyzed by electrophoresis as described earlier. Fragments were visualized with the UV Transilluminator (UVP Inc, San Gabriel, CA, USA) and photographed using the Polaroid MP4 Land Camera (Polaroid Corp, Cambridge, MA, USA). The restriction fragment band positions and sizes of the øX174 DNA/*Hae* III standard marker and isolates were determined using the KODAK 1D 35^{TM} scanner. Four restriction fragments were elucidated for each isolate while the standard marker had 11 fragments (APPENDICES A-D). Each restriction fragment band size (in base pairs [bp]) was rounded up to the nearest whole number and the four added up to give the total size of the PCR product. The total PCR product sizes ranged between 814 and 876bp (APPENDIX E), necessitating the assigning of the restriction profiles to seven groups in the following ranges: Gp. I: 870 - 879bp; Gp. II: 860 – 869bp; Gp. III: 850 – 859bp; Gp. IV: 840 – 849bp; Gp. V: 830 – 839bp; Gp. VI: 820 – 829bp; and Gp. VII: 811 – 820bp (Table 4.5).

3.4.3 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was carried out on twenty-five randomly selected representative isolates (based on their carbohydrate assimilation profiles (whether broad or narrow spectrum) as well as their identification with the ID 32 C Kit as *S. cerevisiae* or otherwise) from five sites within four geographical regions (half of the total sampling site and area) using a modified version of the Clamped Homogenous Electric Fields (CHEF) technique (Chu *et al*., 1986). Cells were harvested from standardized (OD_{600}) 3-day broth cultures by centrifuging (3 ml)(OLE DICH) at 14000 × g for 5 min. Pellets were resuspended in 1 ml EDTA/Tris solution (50mM EDTA (Sigma), 10 mM Tris- HCl (Sigma) pH 7.5), washed 2 times, resuspended in 0.20 ml EDTA/Tris solution containing zymolyase (20 mg ml^{-1} (20T) (Seikagaku Corp, Tokyo Japan) in 10 mM sodium phosphate (Merck)) and held for 15 min at 42 ºC. 0.80 ml portions of 1% LM-Agarose (BMA) was added, gently mixed with a large-bore pipette tip to avoid bubbles and filled into disposable plug molds (BIO-RAD Labs, Hercules, CA, USA) which were held on ice for 15 to 20 minutes. The hardened plugs were pushed out into Eppendorf tubes overlaid with 1ml LET (0.5M EDTA (Sigma), 10 mM Tris (Sigma), pH 7.5) and incubated at 37 ºC for 12 hrs. Plugs were next covered with 1 ml NDS [0.5 M EDTA (Sigma), 10 mM Tris (Sigma), pH 7.5; 1% N-lauroylsarcosine (Sigma), pH 9.5;

2 mg ml^{-1} Proteinase K (USB™ Cleveland, OHIO, USA) added just before use], incubated overnight at 50 ºC, washed 4 times by soaking in 1 ml EDTA/Tris solution for 1 hour each and finally stored at 4 ºC in EDTA/Tris solution until use.

Electrophoresis was carried out on 1% Seakem GTG agarose gels (BMA). One-half of each plug was cut width-wise and loaded into each well. Yeast Chromosome PFG Marker (New England BioLabs) and *Saccharomyces cerevisiae* type strain (CBS 1171) were used as size marker and positive control, respectively with 0.5 × TBE as running buffer. The gels were run in the CHEF DR III system (BIO-RAD) with a voltage of 6 V cm^{-1} for 15 h at a switch time of 60 s followed by 9 h at a switch time of 90 s, all at an included angle of 120 °C. Gels were stained in ethidium bromide (Sigma) (10 mg l^{-1}) for 1 h and rinsed in distilled water with slight agitation on the IKA® KS260 basic shaker (IKA-WERKE GMBH & CO.KG, Staufen, Germany) at 150 rpm for 2 h, the water changed every 30 minutes. The gels were then visualized with the UV Transilluminator (UVP Inc) and photographed (Polaroid).

The chromosome profiles obtained were grouped based on the size and arrangement of bands elucidated by each isolate. One isolate was selected from each grouping for sequence analysis to further resolve their genotypic characteristics. Furthermore, the profiles were normalized and processed for cluster analysis using the BioNumerics Version 2.5 software (Applied Maths, SINT-MARTENS-LATEM, Belgium); based on the Pearson coefficient and the Unweighted Pair Group Method using Arithmetic averages (UPGMA).

3.4.4 Sequence analysis

Sequencing of the mitochondrial gene *COX 2* encoding the cytochrome-*c* oxidase subunit II was carried out on four representative isolates—SH10, T36, T37 and TK13--one each randomly selected from four chromosomal profile groupings based on the size and arrangement of bands elucidated by each isolate. Genomic DNA extraction was performed by pre-growing the isolates on Malt extract-Yeast extract-Glucose-Peptone (MYGP) agar at 25 °C for 5 days. For each yeast, a loop full was incubated in 500µl lysis buffer [2 mM. Tris-HCl (Sigma), 10mM KCl (Merck), 0.3mM $MgCl_2$ (Merck),0.02% (v/v) Triton X-100 (Sigma), 10.0 mg/l Proteinase K (Sigma), 0.617 g/l 1, 4-dithiothreitol (Amersham Pharmacia Biotech, Uppsala, Sweden) at 37 °C for 1 h, boiled for 15 min and centrifuged at 14 000 x g for 2 min. The supernatant was used as template (Petersen *et al*., 2001). PCR amplification of the *COX 2* gene was carried out using the external primers NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) in an automatic thermal cycler (Gene Amp® PCR System 9700, Perkin Elmer) under the following conditions: initial denaturation at 94 °C for 3 min; 36 cycles of 94 °C for 2 min, 42 °C for 1 min and 72 °C for 7 min. The amplified products were purified using the QIAGEN PCR purification kit (QIAGEN, Dorking, UK). For cycle sequencing of the amplicons, the external primers NL-1 and Nl-4 and the internal primers NL-2A (5′-CTT GTT CGC TAT CGG TCT C-.3′) and NL-3A (5′- GAG ACC GAT AGC GAA CAA G-3′) were used. The sequence reactions were performed in an automatic thermal cycler (Gene Amp® PCR System 9700, Perkin Elmer) under the following conditions: initial

denaturation at 96 °C for 2 min, followed by 25 cycles of 96 °C for 30 s, 42 °C for 15 s and 60 °C for 4 min (van der Aa Kühle and Jespersen, 2003). The amplicons produced from *COX 2* genes of the analysed isolates were around 700bp. The sequences were assembled by use of ContigExpress (Vector NTI 7, InforMax, Inc., Frederick, MD, USA). The *COX 2* sequences were compared with available sequences (NCBI) of *S. cerevisiae* CBS1171^{T} (GenBank accession no. AY244992); *S. cerevisiae* S288C (GenBank accession no. AJO11856); *S. paradoxus* Y-17217^{NT} (GenBank accession no. AF442208); *S. bayanus* Y-12624^{T} (GenBank accession no. AF442211); *S. mikatae* Y-27341^{T} (GenBank accession no. AF442209); *S. cariocanus* Y-27337^{T} (GenBank accession no. AF442207); *S. kudriavzevii* Y-27339^{T} (GenBank accession no. AF442210); and *S. pastorianus* Y-27171^{T} (GenBank accession no. AF442212). All sequences were aligned using the ClustalX software (Thompson *et al*., 1997). Five hundred and seventy-nine (579) bp of the *COX 2* genes were used for comparison. The analysed *COX 2* sequences refer to positions 73887 - 74465 on the mitochondria of *S. cerevisiae* S288C.

3.5 Determination of technological properties

3.5.1 Laboratory-scale *pito* fermentation

3.5.1.1 Yeast inocula and fermentation

Two isolates (one with a broad carbohydrate assimilation spectrum and the other with a narrow assimilation spectrum) were selected from each of the ten production sites. The 20 isolates were pre-grown in 20 ml MYGP broth at 30 ºC for 48 h to constitute inocula for the fermentation of *pito* wort obtained from a commercial

producer. The concentration of yeast in the broth at pitching was 10^7 cells ml^{-1}. Fermentation was done in 500 ml screw–cap bottles each containing 200 ml of *pito* wort of pH 3.58. The wort bottles were pitched with the 20 ml broth culture in triplicates and incubated at 30 ºC for 12 h. The bottles were loosely capped to allow the escape of CO_2 during fermentation. The fermentation was conducted at 3-day intervals to allow room for sensory analysis of the final products.

3.5.1.2 Measurement of pH and cell growth

At time 0, 4, 8 and 12 h from start of fermentation, 5 ml samples were drawn out in duplicate for the measurement of pH and cell growth. The pH was determined using the pH meter (JENWAY 3310, Jenway Ltd., Essex, Cambridge, UK), equipped with a glass pH electrode (JENWAY MED BNC-STIK, Jenway Ltd., Essex, Cambridge, UK). The pH meter was calibrated against standard buffer solutions (Bie & Berntsen A-S, Rødovre, Denmark) at pH 4.0 and 7.0 prior to its use. Cell growth was determined in total counts by microscopy (OLYMPUS CH30, Olympus Opt. Co. Ltd, Tokyo, Japan) and a counting chamber (Bright-Line Haemacytometer, REICHERT, Buffalo, NY, USA).

3.5.1.3 Sensory analysis

Sensory analysis was conducted on all 20 *pito* samples produced after 12 hours of fermentation. A 5-member untrained panel of adult males familiar with *pito* was used to evaluate the quality attributes of the experimental *pito* against the commercially prepared product. Panelists were requested to score mouth feel, taste,

and aroma using a 3-point Hedonic scale (Score: 3 = very similar to; 2 = quite similar to; 1 = different from; commercial *pito*). Tests were conducted three times, independently at 3-day intervals. Mean scores ± standard deviations for each quality attribute of *pito* produced by each strain were represented in bar charts.

3.5.1.4 Yeast flocculation

Flocculence behavior of *S. cerevisiae* isolates was investigated using the Burns' Test (Burns, 1937). The isolates were propagated in 200 ml MYGP broth for three days at 30 ºC and cells were harvested by centrifugation (Hettich Universal 30RF, Tüttingen, Germany) at 3000 × g for five minutes. The pellet, after removal of the upper dark layer with a spatula, was reconstituted and washed four times in 10 ml Washing solution (0.65 g $CaSO_4.2H_2O$ (Merck) in 1000 ml Milli-Q water) in 13 ml Sarstedt tubes (SARSTEDT, Aktiengeselischaft & Co, Nümbrecht, Germany) with centrifugation as described earlier. The pellet was reconstituted in 10 ml Solution C (mixture of 375 ml Solution A (0.86 g $CaSO_4.2H_2O$ (Merck)) in 1000 ml Milli-Q water), 50 ml Solution B (34.0 g $CH_3COONa.3H_2O$ +20.25 g CH_3COOH (Merck), added Milli-Q water up to 500 ml) and Milli-Q water up to 500 ml, pH 4.5 ± 0.1) in graduated tapering glass. The tapering glasses with their contents were held in a water bath (Grant Instruments (Cambridge) Ltd, Cambridge, England) at 20 ºC for 20 min. to stabilize the temperature of the suspensions. With the spout firmly closed with the thumb to avoid spillage, each tube was briskly but gently agitated to homogenize its contents. Tubes were left to stand and flocculation (in ml)

determined at time periods of 2, 4, 6, 8, 10, 15, 20, 30, 40, 50 and 60 min. By this sedimentation test, one of two characteristic patterns of sedimentation could be observed; for flocculent yeast the suspension quickly separated into two layers with a fairly distinct boundary near the top of the tube. The boundary settled rapidly and it was the position of this falling boundary that was observed and recorded. The yeast concentration was low above the boundary, and high below it. For non-flocculent yeast, the boundary formed much more slowly near the bottom of the tube and gradually rose. The tests were conducted in duplicate for each isolate and mean values plotted against time to demonstrate flocculence behavior of the isolates.

3.5.1.5 Aroma analysis

Pito produced with each of ten out of the twenty yeast strains from Ghana used for the earlier investigations (selected based on various technological properties exhibited viz. cell growth, flocculence behavior, and organoleptic quality of *pito* produced) and three from Burkina Faso (randomly selected based on their carbohydrate assimilation spectra) was analyzed by headspace, for its aroma constituents. Samples were held at -20 ºC prior to analysis. For extraction, a mixture of 200 ml *pito* (supernatant) and 1 ml internal standard solution of 4-methyl-1-pentanol (50 ppm in water) (Aldrich, Milwaukee, WI, USA) in a 500 ml gas washing bottle with a magnetic bead was equilibrated to 30 ºC in a water bath (Grant Instruments) placed over a magnetic stirrer (Selecta, Buch and Holm A/S.

Herlev, Denmark) for 10 minutes. Nitrogen gas was bubbled through the bottle at a flow rate of 200 ml min^{-1} and out through a trap consisting of a glass tube (6.8 × 0.4 cm) containing Porapak Q (Waters Corp., Milford, MA, USA) (50 – 80 mesh) which had been previously cleaned with pure diethyl ether (Bie & Berntsen) for 60 minutes. Volatiles adsorbed onto the Porapak Q columns were eluted with diethyl ether. Eluents were concentrated by evaporation with N_2 gas stream to 100 mg and stored at -20 ºC until use in GC-MS analysis.

A Hewlett-Packard G1098A GCD System (GC-MS, Hewlett-Packard, Palo Alto, CA, USA) equipped with a Hewlett-Packard DB-WAX (DB122-7032) column (30 m x 0.25 μm [internal diameter] x 0.25 mm film thickness) was used to analyze the volatile eluents. Two microlitres of eluent were injected (split ratio 1:20) using the following temperature program: 10 min at 40 °C, increased to 240 °C at 6 °C min^{-1}, and held constant at 240 °C for 30 min. Identification of aroma compounds was determined in the Total ion mode scanning a mass to charge ratio (m/z) range of between 25 and 550. Further identification was obtained by probability-based matching with mass spectra in the G1033A NIST PBM Library (Hewlett-Packard) containing 75,000 reference spectra.

3.6 Statistical analysis

Data generated from determination of pH change and cell growth during fermentation as well as sensory (quality) attributes of Dagarti *pito* produced by the strains was analyzed using the Statistical Analysis System (SAS, Release 8.2, NC,

USA). One-way analysis of variance (ANOVA) was carried out to determine the significant error margin (SEM) between various independent means ($p < 0$).

CHAPTER FOUR

4.0 RESULTS

4.1 Study area and origin of isolates

The cities and towns from which sampling took place are marked within their geographical locations in the political map of Ghana (Plate 3.1). Nyankpala is considered a satellite town of Tamale within the Northern Region while Monaco and Ayigya are both suburbs of Kumasi in the Ashanti Region.

Based on interviews with Mary Benyima of Cultural Centre Pito Bar, Tamale and Mrs. Jimmah of Nyankpala, corroborated by accounts from other producers in other parts of the country, a flow diagram of the process of Dagarti *pito* production was elaborated as in Fig. 4.1.

4.2 Phenotyping of yeast isolates from dried *pito* yeast

Yeast populations ranged between 10^6 and 10^8 colony forming units per gram of dry yeast sampled from the ten locations. A total of 249 yeast isolates were obtained (25 from each site of which one was lost during sub-culturing). Of these 247 isolates (99%) showed macromorphological and micromorphological characteristics typical of *S. cerevisiae* (Group A), while 2 isolates (1%) showed different characteristics (Group B) as shown in Table 4.1 below.

Table 4.1: Morphological Characteristics of Isolates

Group	No. of Isolates	Characteristic	
		Colony Morphology	Cell Morphology
A	247	Cream, small/large, round, smooth/glistening, mucoid, flat/raised, opaque, entire	Spherical/globose, multilaterally budded
B	2	Dirty-white, smooth/glistening, flat/raised, opaque	Ellipsoidal/elongated, multilaterally budded

Determination of carbohydrate assimilation profiles with the API ID 32C Kit revealed 58 different profiles [Tables 4.2 (a) and 4.2 (b)], and resulted in a split in group A (Table 4.1) which was made up of 247 isolates with similar morphological characteristics, into two sub-groups-- A (i) and A (ii) (Table 4.3). Sub-group A (i) had 179 isolates (72%) with 53 broad-spectrum assimilation profiles (isolates could assimilate more than three carbohydrates), and were clearly identified in API galleries as *S. cerevisiae* while sub-group A (ii) comprised 68 isolates (27%) with 4 assimilation profiles (3 narrow [isolates could assimilate three or less carbohydrates] and 1 broad spectrum) atypical of *S. cerevisiae* and could not be clearly identified. Two isolates (1%) in Group B, which had macromorphological and micromorphological characteristics atypical of *S. cerevisiae*, and a broad-

spectrum assimilation profile, were identified as *Candida kefyr* (Table 4 .3). These initial findings contrast with those of van der Aa Kühle *et al*. (2001) who reported that 45% of 100 yeast isolates from sorghum beer produced in Ghana and Burkina Faso

Table 4.3: Carbohydrate Assimilation Profiles of Isolates determined by API ID 32 C Kit

Carbohydrates	Group		
	A (i) *S.cerevisiae* (179 isolates)	**A (ii)** Not readily identified as *S. cerevisiae* (68 isolates)	**B** *Candida kefyr* (2 isolates)
Galactose	70/179	-	2/2
Actidione	-	-	-
Saccharose	147/179	-	2/2
N-acetyl-glucosamine	2/179	-	2/2
DL-lactate	50/179	-	2/2
L-arabinose	-	-	-
Cellobiose	3/179	-	-
Raffinose	58/179	-	2/2
Maltose	177/179	61/68	2/2
Trehalose	75/179	-	2/2
2-Keto-gluconate	-	-	-
ά-Methyl-D-glucoside	73/179	-	2/2
Sorbitol	-	-	-
D-Xylose	-	-	-
Ribose	1/179	-	-
Glycerol	-	-	-
Rhamnose	-	-	-
Palatinose	123/179	1/68	2/2
Erythritol	-	-	-
Melibiose	-	-	-
Melezitose	90/179	2/68	2/2
Gluconate	-	-	-
Levulinate	-	-	-
Mannitol	-	-	-
Lactose	-	-	-
Inositol	-	-	-
Glucose	179/179	68/68	2/2
Sorbose	-	-	-

Glucosamine	-	-	-
Esculin	-	-	-

A(i)—isolates with carbohydrate assimilation profiles which could be easily determined by API ID 32 to belong to *Saccharomyces cerevisiae* (according to Vaughan-Martini and Martini, 1998); **A (ii)—**isolates with carbohydrate assimilation profiles atypical of *Saccharomyces cerevisiae* and therefore could not be identified in API galleries.

were identified as *S. cerevisiae* whereas 38% had physiological properties atypical of *S. cerevisiae* or any other member of the complex *sensu stricto,*

Table 4.4: Distribution of typical and atypical *Saccharomyces cerevisiae* carbohydrate assimilation profiles among sampling sites.

Typical *S .cerevisiae* profiles		
Profile(s)	**No.**	**Sampling site(s)**
11,12,20,21,22,27,33,40,41,42,43,44,54	13	Accra
18,31,37,38,47,48,53,57	8	Ayigya-Kumasi
28,34,50	3	Cape Coast
8,25,30,56	4	Tamale
13,14,15,36	4	Takoradi
19,32,39	3	Monaco-Kumasi
6,24,52	3	Sunyani
17	1	Ho
4	1	Monaco-Kumasi, Suhum
7	1	Takoradi, Ho, Suhum
9	1	Cape Coast, Ayigya & Monaco(Kumasi), Sunyani
10	1	Monaco-Kumasi, Nyankpala
16	1	Takoradi, Nyankpala, Suhum
23	1	Cape Coast, Takoradi, Tamale , Sunyani
26	1	Nyankpala , Ho
29	1	Ayigya-Kumasi , Accra
35	1	Takoradi, Sunyani , Ho
45	1	Cape Coast, Sunyani
46	1	Sunyani, Tamale
49	1	Accra, Ho
51	1	Takoradi, Sunyani, Cape Coast, Tamale, Ho
55	1	Sunyani, Takoradi
Sub-total	**53**	
Atypical *S.cerevisiae* profiles		
3,5	2	Cape Coast

1	1	Monaco-Kumasi, Nyankpala
2	1	Monaco-Kumasi, Cape Coast, Takoradi, Sunyani, Ho, Tamale, Nyankpala
Sub-total	**4**	

Typical *S. cerevisiae* assimilation profiles comprised glucose, maltose and other carbohydrates; atypical *S. cerevisiae* assimilation profiles comprised only glucose (**1**); glucose and maltose (**2)**; glucose and melezitose (**3)**; glucose, melezitose and palatinose (**5).**

as they were able to assimilate c[illegible]e, maltose and ethanol as carbon sources. As depicted in Table 4.4, the most common profile of assimilating only glucose and maltose (Profile 2), atypical of *S. cerevisiae* though, was seen for 61 isolates from 7 sampling sites in Monaco-Kumasi (4), Cape Coast (3), Takoradi (12), Sunyani (3), Ho (16), Tamale (9) and Nyankpala (14). Thirty-nine of the typical *S. cerevisiae* profiles were exclusive to 8 sampling sites in the following numbers: Accra (13); Ayigya-Kumasi (8); Cape Coast (3): Tamale (4); Takoradi (4); Monaco-Kumasi (3); Sunyani (3); Ho (1) while two atypical profiles (3 and 5) were exclusive to Cape Coast. Each of the remaining 15 profiles (14 typical and 1 atypical of *S. cerevisiae*) was however shared by 2 or more sampling sites. No profile was exclusive to Nyankpala or Suhum.

Table 4.5: Restriction profiles of forty-two dry *pito* yeast isolates from 10 different production sites in eight geographical regions of Ghana.

ITS-PCR Group*	No. of Isolates	Band Size (bp)	Restriction Fragments	Identification
I	5	875 ± 0.75	316+238+182+139	*S. cerevisiae*
II	9	864 ± 2.86	310+237+180+137	*S. cerevisiae*
III	10	855 ± 2.65	309+235+178+133	*S. cerevisiae*
IV	9	847 ± 2.28	308+234+175+130	*S. cerevisiae*

V	4	837 ± 2.38	307+233+175+122	*S. cerevisiae*
VI	1	828	304+231+172+121	*S. cerevisiae*
VII	4	817 ± 1.48	303+230+168+115	*S. cerevisiae*

* Group I: AC15, HO5, HO6, HO7, HO17; Group II: AC14, AC17, AC24, CC24, TK13, HO2, SH3, SH9, SH10; Group III: A9, A15, M8, M11, M22, AC7, CC10, CC16, TK5, TK25; Group IV: A5, A19, M7, M17, CC3, TK24, SH7, T14, T30; Group V: TK2, SY4, N19, N22; Group VI: N1; Group VII: SY2, SY14, SY15, SY18. Band size values for Groups I-V and VII represent means ± standard deviations of band sizes of members of each group (APPENDIX E).

4.3 Genotyping of Isolates

4.3.1 ITS-PCR+ RFLP

Forty-two out of the fifty isolates characterized by amplification of the ITS1-5.8S rDNA-ITS2 region followed by restriction analysis with *Hae* III endonuclease produced a pattern of restriction profiles similar to that of the *S. cerevisiae* type strain CBS 1171. The ITS regions varied in size from 816 – 875 base pairs (bp). For most isolates, four restriction bands were observed with band sizes from 115 to 316 bp as shown in Figs. 4.2(a) - 4.2(d) and confirmed with the KODAK ID 35^{TM}(APPENDICES A-D). The profiles were shared by both isolates with narrow and broad assimilation spectra. These results compare favourably with a study by Naumova *et al.* (2003) on the molecular genetic identification of *Saccharomyces sensu stricto* strains from African sorghum beer which demonstrated that ITS-PCR+RFLP analysis with the endonucleases *Hae*III, *Hpa* II, *Scr*FI and *Taq* I was useful for discriminating *S. cerevisiae, S. kudriavzevii, S. mikatae* from one another and from the *S. bayanus/S. pastorianus* and *S. cariocanus/S. paradoxus* pairs. That study also reported that all the sorghum beer strains showed the same molecular size of about 850 bp and exhibited the same restriction patterns of four bands as the type culture of *S. cerevisiae* CBS 1171, using *Hae*III endonuclease with band sizes

from 125 to 325 bp. An earlier study by Esteve-Zarzoso *et al*. (1999) also found the molecular size of the PCR product of *S. cerevisiae* [Spanish Type Culture Collection (CECT) 1942^{T}, 1971] to be 880 bp, while the restriction fragments produced using the endonuclease *Hae* III numbered four, ranging from 150 to 320 bp. Three isolates—A13, CC8 and SH2 did not show

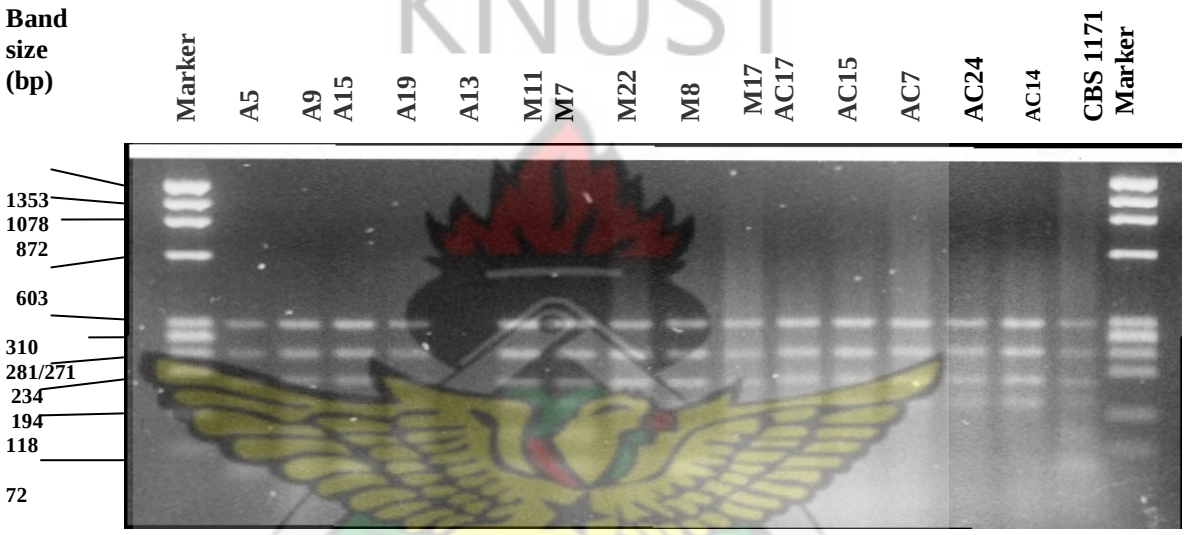
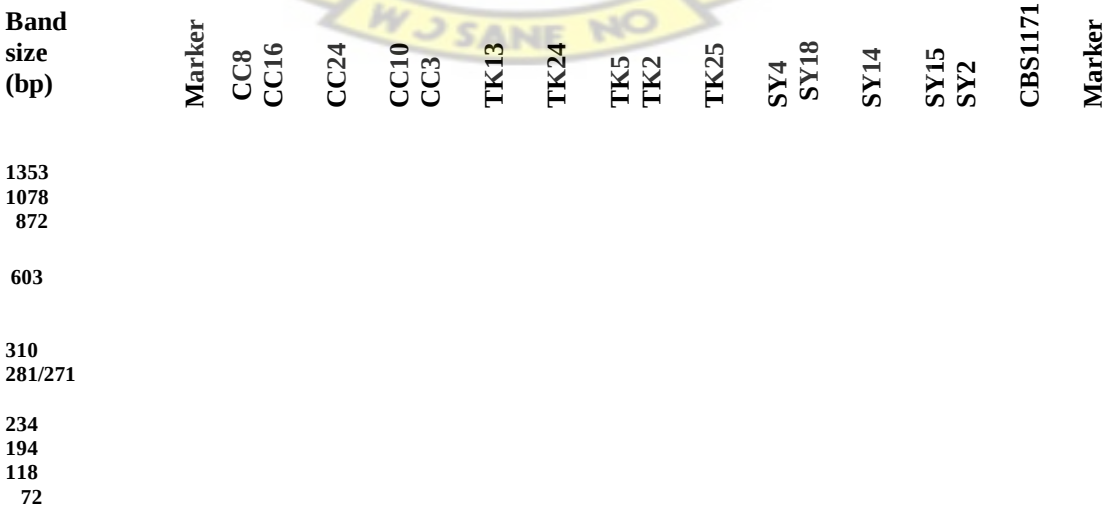


Fig.4.2 (a): Restriction fragments of isolates from Ayigya (A), Monaco (M) and Accra (AC). Almost all isolates had four restriction bands with various band size ranges as shown in Table 4.5: 130 – 308bp (A5, A19, M7, M17) [Gp.IV]; 133 -309bp (A9, A15, M8, M11, M22, AC7) [Gp.III]; 137 -310bp (AC14, AC17, AC24) [Gp.II] and 139 – 316bp (AC15) [Gp. I]. Fragments outside these are not true bands. A13 did not show any restriction fragments (See Appendix A). Molecular mass marker Ø x174 DNA/*Hae* III (Promega) is indicated in base pairs.



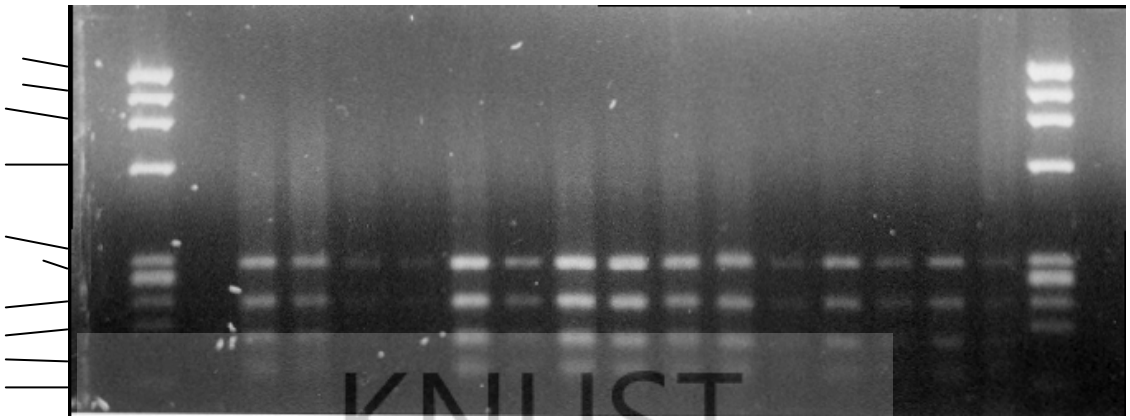


Fig.4.2 (b): Restricted fragments of isolates from Cape Coast (CC), Takoradi (TK)

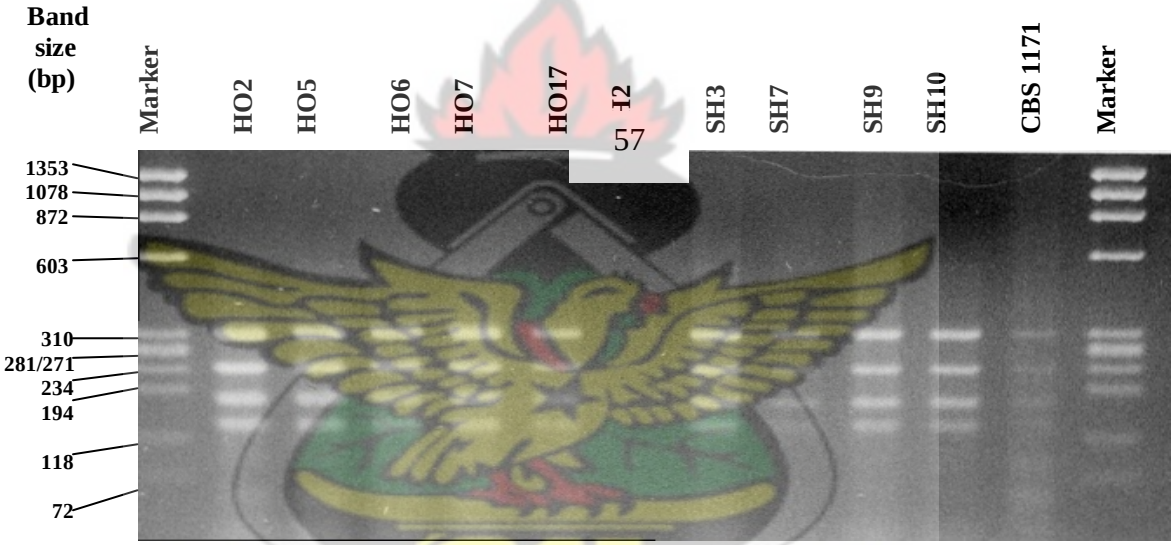
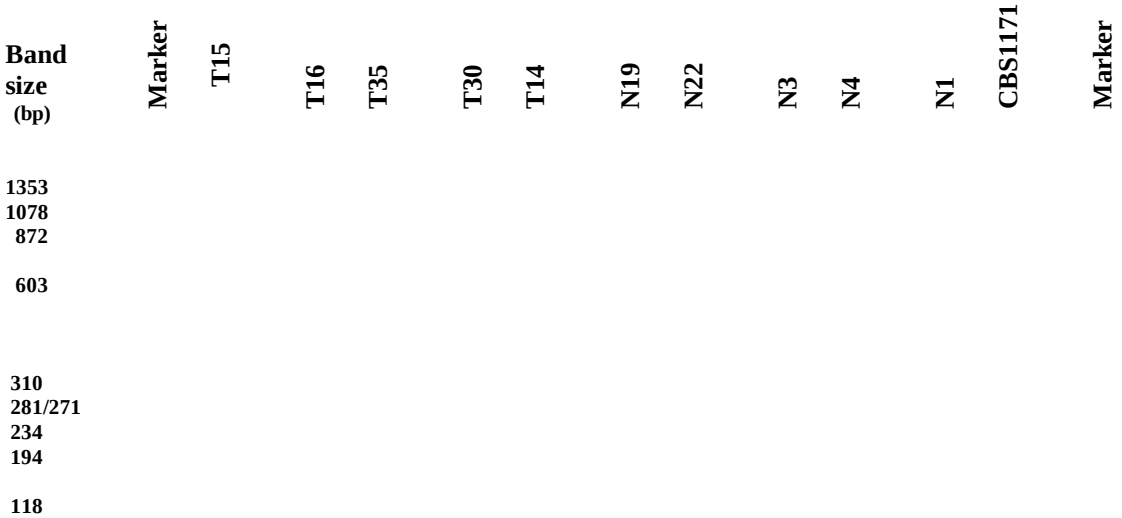


Fig.4.2 (c): Restricted fragments of isolates from Ho (HO) and Suhum (SH). Almost all isolates had four restriction bands with various band size ranges as shown in Table 4.5: 130 – 308bp (SH7) [Gp.IV]; 137 -310bp (SH3, SH9, SH10, HO2) [Gp.II] and 139 – 316bp (HO5, HO6, HO7, HO17) [Gp. I]. SH2 did not show any restriction fragments. Molecular mass marker Ø x174 DNA/*Hae* III (Promega) is indicated in base pairs.



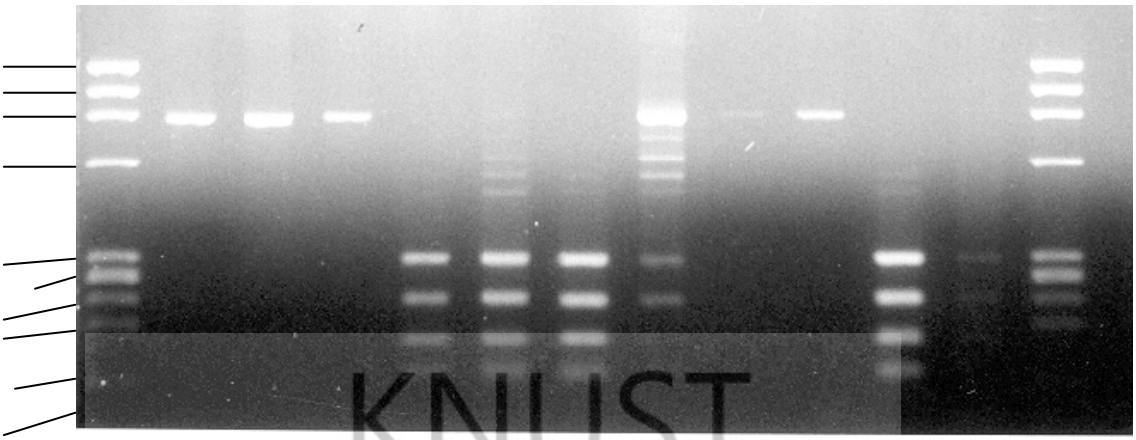


Fig.4.2 (d): Restricted fragments of isolates from Tamale (T) and Nyankpala (N). A number of isolates had four restriction bands with various band size ranges as shown in Table 4.5: 121 – 304bp (N1) [Gp. VI); 122 – 307bp (N19, N22) [Gp. V] and 130 – 308bp (T14, T30) [Gp.IV] while N19 and N22 had extra fragments. Fragments outside these are not true bands (See Appendix D). T15, T16, T35, N3 and N4 could not be digested. Molecular mass marker Ø x174 DNA/*Hae* III (Promega) is indicated in base pairs.

any restriction fragments while isolates T15, T16, T35, N3 and N4 showed chromosomal DNA (PCR products) that were not digested during restriction.

Isolates N22 and T14 possessed addit riction fragments. This is an aberrant pattern as *Hae* III endonuclease is known to cleave the PCR product (DNA) of members of the *Saccharomyces* sensu stricto complex into three or four bands during restriction (Esteve-Zarzoso *et al.*, 1999; Naumova *et al.*, 2003).

4.3.2 Pulsed Field Gel Electrophoresis (PFGE)

Chromosome profiles obtained by PFGE for isolates were typical of *S. cerevisiae* as most isolates possessed 16-band chromosomes with sizes ranging from 200 to 1900 kilo base pairs (kbp) (Fig.4.3). For all isolates, the chromosomes were significantly larger than the standard marker. The DNAs of the smallest chromosomes (I, VI, III, and IX) appear to have resolved well in all isolates. In most of the isolates (A9, A15, M11, AC17, AC15, AC7, AC24, AC14, CC8, CC16,

CC24, CC10, CC3, TK13, TK24, TK5, TK2 and TK25), chromosomes V and VIII were separated or almost separated as for *S. cerevisiae* type strain CBS 1171. Some isolates, however showed chromosome length polymorphism both by size and number of bands. Chromosomes II and V were absent in isolate A5 whereas isolate M17 did not appear to possess chromosome VIII. Chromosome X was absent in isolates M22 and M8. Chromosomes XII and IV appear as single bands in all isolates except M7, M22, M8, M17, CC16 and CC24 whereas chromosomes VII and XV appear as single bands in all isolates. A separate chromosome XI is seen in all isolates. Two co-migrating doublets consisting of chromosomes XIII, XVI and VII, XV respectively, were closer to each other in isolates A19 and A13 than in the other isolates.

Figure 4.4 is a dendrogram showing the clustering of the electrophoretic patterns of 25 *Saccharomyces cerevisiae* strains isolated from Dagarti *pito* yeast. No correlation was seen between the clustering and the assimilation spectra of isolates as clusters were formed between isolates with broad and narrow assimilation spectra.

Two large (II and III) and three small (I, IV and V) clusters were generated from the normalized and processed chromosome profiles. Clusters II and III comprised 8 and 12 isolates, respectively, of both narrow and broad assimilation spectra, from three sites-Monaco-Kumasi (M), Ayigya-Kumasi (A) and Accra (AC). Cluster I had two isolates which came from two sites—Monaco-Kumasi (M) and Takoradi (TK). Clusters IV and V comprised two and one isolates respectively, all of which

came from the same site—Ayigya-Kumasi (A). Whereas isolates from the Monaco-Kumasi site (M) were in the majority (50%) in cluster II, they were not represented at all in cluster III, which had majority (42%) of isolates from Cape Coast (CC). Isolates from Accra (AC) were common to both clusters II and III in the same proportion (25%). The two large clusters (II and III) merged at 73% similarity but had similarities between 50% and 60% with the smaller clusters (I, IV and V).

III

IV

V

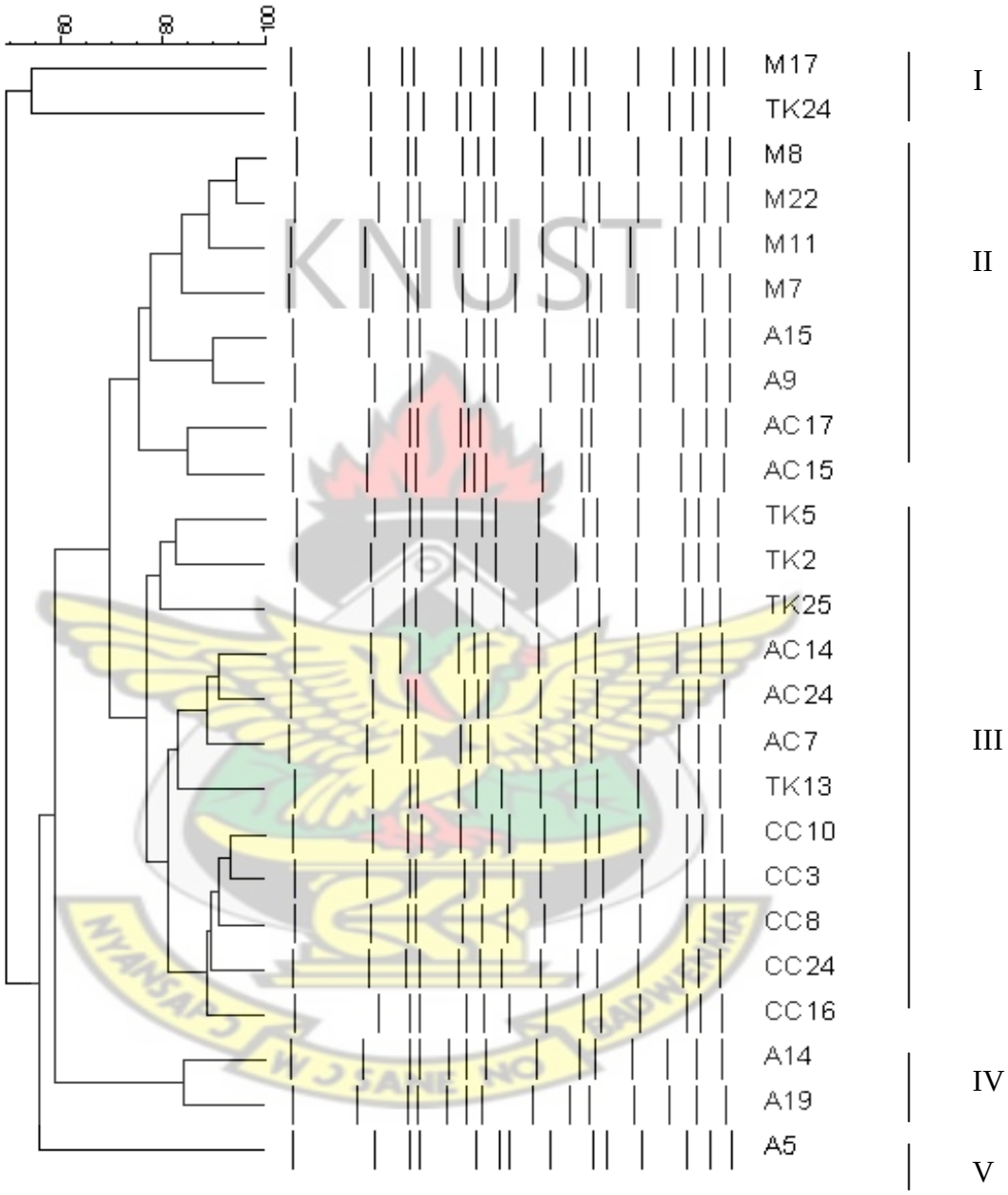


Fig. 4.4: Dendrogram showing the clustering of 25 *Saccharomyces cerevisiae* strains isolated from Dagarti *pito* from five sampling sites within four geographical regions of Ghana—Ayigya-Kumasi (A), Monaco-Kumasi (M), Accra (AC), Cape Coast (CC) and Takoradi (TK)

4.3.3 Sequencing

Sequencing of the *COX2* genes of four representative isolates confirmed earlier indications that all isolates are strains of *S. cerevisiae,* irrespective of their phenotypic characteristics. Besides showing strong homology (≥ 98.8 %) among themselves, each of the isolates exhibited very close similarity with members of the *Saccharomyces sensu stricto* complex, particularly *S. cerevisiae* type strain CBS1171(98.7%[in average of 98.6, 98.4, 98.6 and 99.3]) (Table 4.6).

4.4 Technological properties of isolates

4.4.1 Change in pH and cell number during fermentation

Initial pH of *pito* wort was in the acidic range and averaged 3.58. Between times 0 and 12h, wort fermentation by each of the 20 isolates selected for fermentation studies resulted in very little change in pH with values ranging between 3.39 and 3.55 (Table 4.7). Increase in yeast cell numbers over the 12 h period of fermentation was observed for most of the isolates (Table 4.8). Majority (13) out of the 20 isolates of *S. cerevisiae* (7 in Group II) and 6 in Group III) exhibited exponential growth within 4-12 h of fermentation. Four isolates in Group I showed minimal increase in growth over the entire period while three isolates in Group IV showed decreasing cell numbers in suspension after 8 h (Fig.4.5). Isolates showing the fastest growth mainly belonged to the phenotypes with the broad-spectrum assimilation patterns.

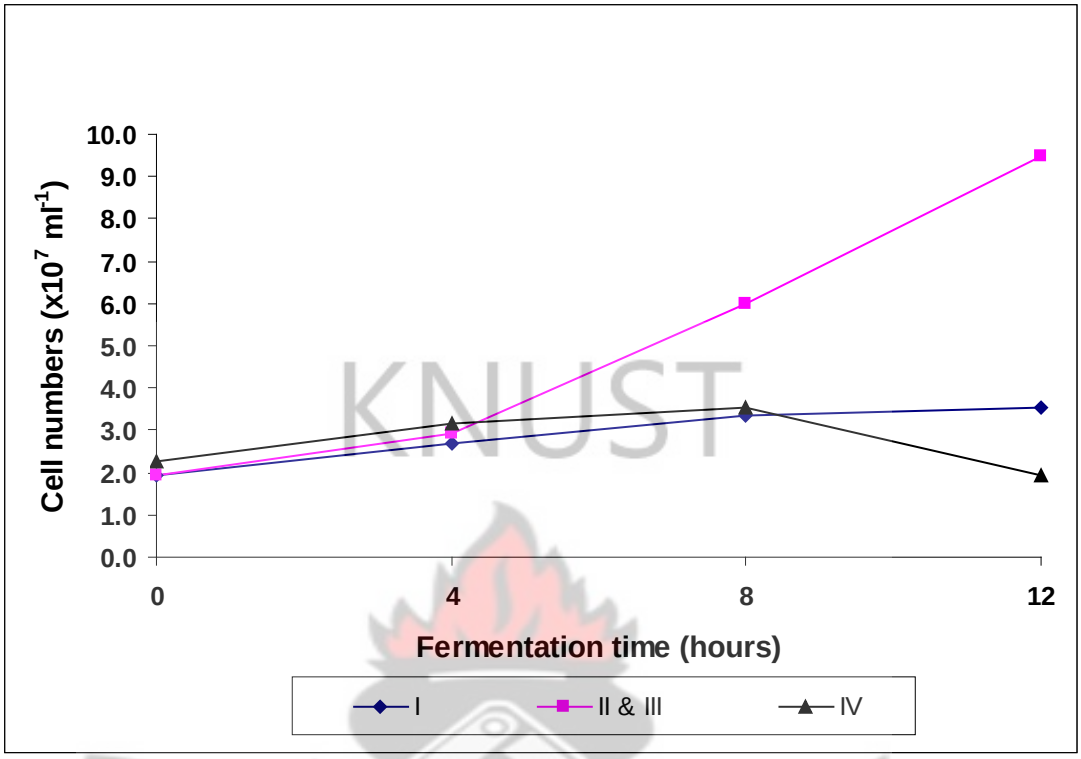
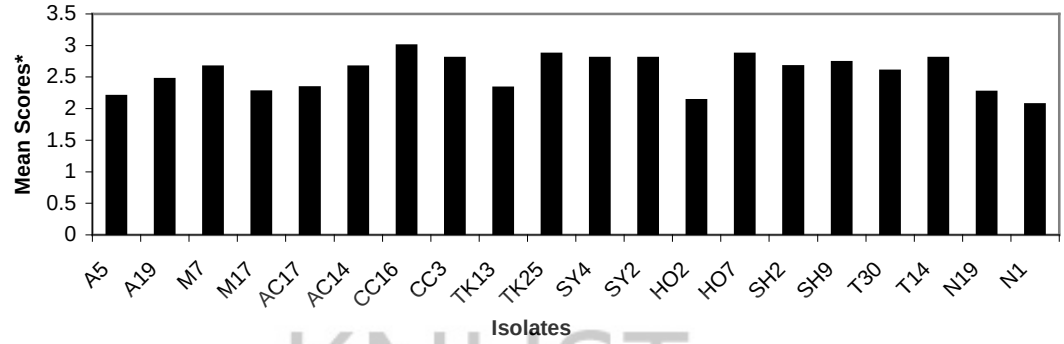


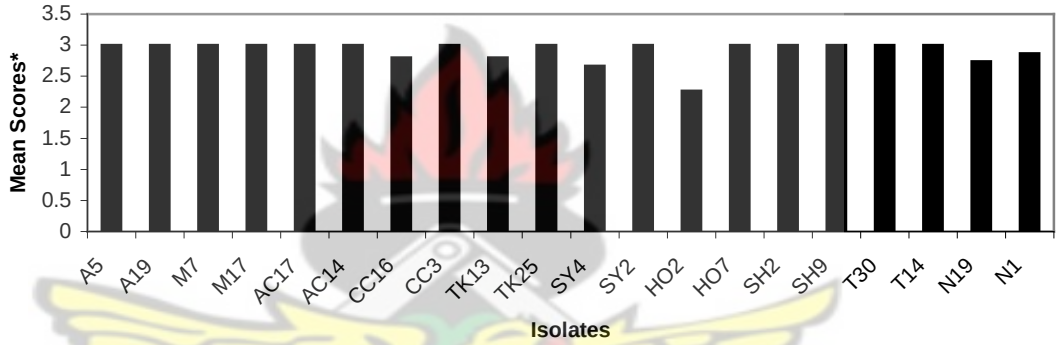
Fig.4.5: Growth pattern of isolates. Gp .I -- isolates with minimal growth throughout fermentation period; Gp. II –isolates with substantial growth after 4 hours of fermentation; Gp. III –isolates with significant growth after 8 hours of fermentation; Gp..IV –isolates showing decrease in growth after 8 hours.

4.4.2 Sensory Analysis

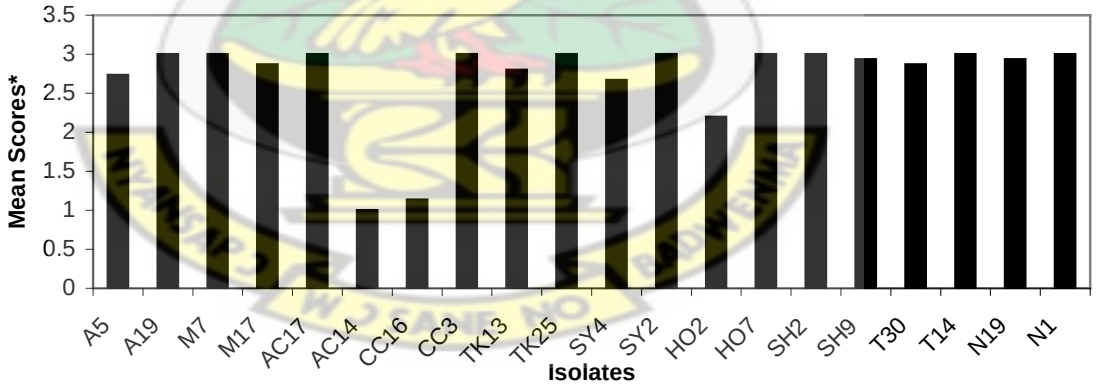
Pito produced with the isolates showed variations in the three sensory attributes of mouth feel, taste and aroma (Fig.4.6). Only *pito* produced with isolate CC16 had mouth feel very similar (score 3), to commercial *pito*; all others fell between quite similar and very similar (scores 2 and 3). In terms of taste, fourteen isolates from nine sites--A5, A19, M7, M17, AC17, AC14, CC3, TK25, SY2, HO7, SH2, SH9, T30 and T14-- produced *pito* of very similar taste as the commercial product (score 3) while



A



B



C

Fig 4.6: Quality attributes of *pito* produced with isolates. A---Mouth Feel; B---Taste; C---Aroma. *Scores represent means of three independent evaluations made in triplicate by five panelists with analysis of variance (ANOVA) at $p<0.05$, using the SAS programme (SAS. Release 8.2, NC, USA).

Pito produced with each of isolates A19, M7, AC17, CC3, TK25, SY2, HO7, SH2, T14 and N1 had aroma very similar to that of the commercial product (score 3). *Pito*

produced with isolates AC14 and CC16 had virtually different aroma (scores b/n 1 and 1.5) in contrast to near very similar mouth feel and taste (scores b/n 2.6 and 3) as the commercial product. A strain from Ho (HO2) produced *pito* with all three attributes quite similar (score 2) to those of the commercial product ($p<0.05$).

4.4.3 Yeast flocculation

As seen from Fig.4.7 below, the majority of the investigated isolates (17 out of 19) belonged to non-flocculent Groups II (AC14, AC17 and CC16) and III (A19, M7, M11, CC3, TK13, TK25, SY4, SY2, HO7, SH2, SH9, T30, N19, and N1).

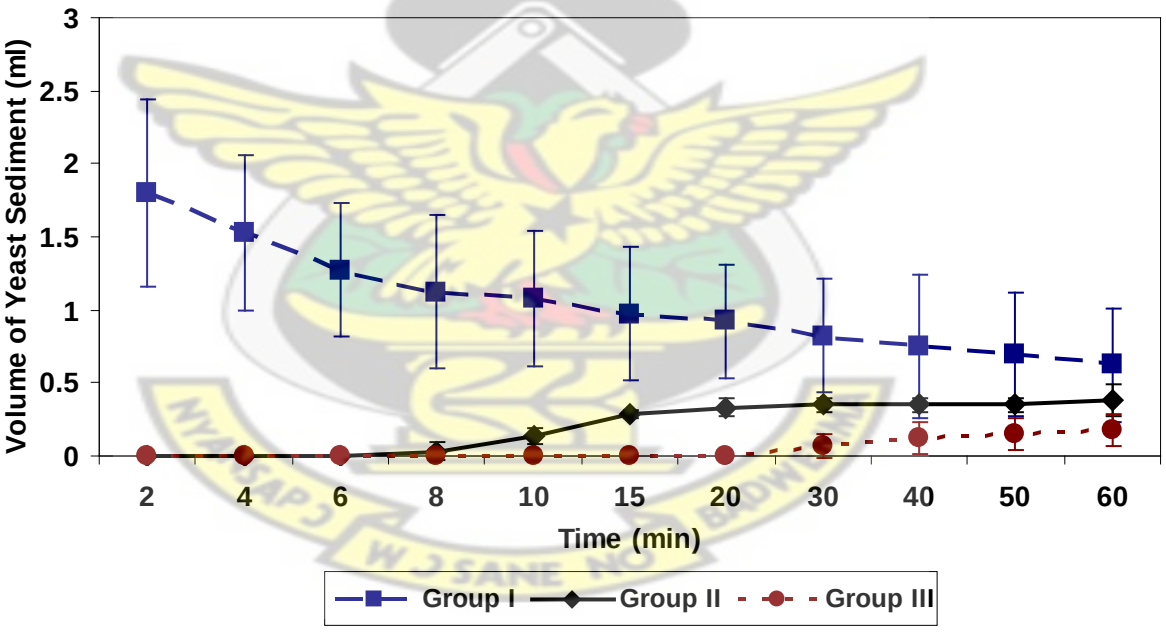


Fig. 4.7: Flocculence behavior of 19 *pito* isolates from eight geographical regions of Ghana. Values represent means of readings taken in triplicate with standard deviations.

Only two out of the nineteen isolates investigated (A5, T14) belonging to Group I were flocculent with sediment volumes of 0.75 ± 0.21 and 1.4 ± 0.00, respectively

after 10 minutes (Table 4.9). These isolates, which came from different sites, also differed in their flocculation patterns, one sedimenting at the bottom (bottom-fermentor) and the other forming clumps at the surface of the suspension (top-fermentor) in the fermentation trials.

4.4.4 Aromatic compounds produced by isolates

As shown in Table 4.10, all ten Ghanaian isolates possessed the ability to form aromatic compounds representing the alcohols, esters, and ketones. Whereas they all produced varying amounts of ethanol, 1-Propanol, 2-methyl-propanol, and 3-methyl-butanol, two (A5 and M7) did not produce 1-Propanol. Only isolates CC3, HO7, T14, and N19 produced the alcohol 4-Pentenol. Ethyl octanoate was the most commonly produced ester (9 out of 10 Ghanaian isolates--A5, M7, AC17, CC3, TK25, SY2, SH9, T14, N19) while isobutyl acetate and ethyl propionate were the least produced (1 Ghanaian isolate each--SY2 and CC3 respectively). Nine isolates (A5, M7, AC17, CC3, TK25, SY2, SH9, T14, N19) produced the ketone—1-(4-ethylphenyl)-Ethanone, followed by 7 isolates (A5, M7, AC17, CC3, TK25, SY2, T14) which produced 1, 1'- (1, 4-phenylene) bis-Ethanone. 2'hydroxy-4', 5'-dimethylacetophenone was produced by only isolate SY2. The aldehyde—4-ethyl-Benzaldehyde--was produced by only isolate A5. Isolate HO7, which produced all the alcohols, did not produce detectable esters and ketones. In contrast, all three isolates from Burkina Faso *dolo* yeast produced the alcohols, ethanol, 1-Propanol, 2-methyl-propanol, 3-methyl-butanol and 4-Pentenol. Only one of them, Z14,

produced varying amounts of the esters--ethyl propionate, isobutyl acetate, isopentyl acetate, ethyl hexanoate and ethyl octanoate while ethyl decanoate was produced by Z14 and S18. None of the three isolates could produce isoamyl acetate. The ketone 1-(4-ethylphenyl)-Ethanone was produced by all three isolates while only isolate S18 produced 1, 1'- (1, 4-phenylene) bis-Ethanone. None of the Burkina Faso isolates produced a detectable aldehyde.

CHAPTER FIVE

DISCUSSION

Out of a total of 249 yeast isolates obtained for the study, 247 isolates (99%) from nine sites were identified as *Saccharomyces cerevisiae*, while 2 isolates (1%) from one site were identified as *Candida kefyr*. This implies an almost exclusive presence of *S. cerevisiae* in all but one of the ten *pito* production sites sampled in eight geographical regions of Ghana.

This finding differs from earlier ones by Demuyakor and Ohta (1991) who reported *S. cerevisiae* as being the predominant species (33%) in Ghanaian *pito*, as well as a high prevalence of *Candida* spp. (17%) and *Kluyveromyces* spp. (23%), along with members of four other genera—*Torulopsis, Pichia, Zygosaccharomyces* and *Trichosporon*; and Sefa-Dedeh *et al*. (1999) who also reported the co-existence of *S. cerevisiae* (38%) with species of six other genera-- *Candida tropicalis* (19%), *Torulaspora delbrueckii* (14%), *Kloeckera apiculata* (9.5%), *Hansenula anomala*

(9.5%), *Schizosaccharomyces pombe* (4.75%) and *Kluyveromyces africanus* (4.75%). A prevalence of *S. cerevisiae* of not more than 38% of the yeast population as well as high levels of several other genera was demonstrated in these studies. These reports, even though agreeing on the dominance of *S. cerevisiae*, were based on identification methods involving physiological and biochemical tests which, according to Casey *et al*. (1990) and Querol *et al*. (1992), are inadequate in identifying yeasts, particularly at the subspecies level.

The findings of the current study, however, agree with those of Konlani *et al*. (1996) who found that *S. cerevisiae* accounted for 55-90% of the yeast population in *pito* yeast samples from five out of six production sites in Togo and Burkina Faso while *Candida krusei* was dominating (70%) in the last sample. Furthermore, van der Aa Kühle *et al*. (2001) also reported the almost exclusive (99%) occurrence of *S. cerevisiae* in dry yeast from sorghum beer (*pito*) production sites in the northern part of Ghana and Burkina Faso. This concurrence is justified by the fact that the reported studies of Konlani *et al*. (1996) and van der Aa Kühle *et al*. (2001), just like the present one, used identification methods based on molecular techniques including Intergenic Transcribed Spacers-Polymerase Chain Reaction + Restriction Fragment Length Polymorphism (ITS-PCR + RFLP), Pulsed-Field Gel Electrophoresis (PFGE) and DNA sequencing. These techniques have been found valuable in identifying and characterizing yeasts by Kurtzman and Robnett (1998). They have also been reportedly used successfully in differentiating between several yeast species including *Saccharomyces* spp. (Guillamón *et al*., 1998; Esteve-

Zarzoso *et al.,* 1999), as well as among strains of *Saccharomyces* spp. within both wine and brewing yeast, brewing contaminants and baker's yeast (Casey *et al.*, 1990; Bidenne *et al*., 1992; Vezinhet *et al.*, 1992 and Jespersen *et al*., 2000). Their use in subspecies typing of *S. cerevisiae* has also been reported by Baleiras Couto *et al*. (1996) and Naumova *et al.* (2002). DNA sequencing of the mitochondrial gene *COX2* using the methods described by Petersen *et al*. (2001) and van der Aa Kühle and Jespersen (2003) have helped to establish the close relatedness of the isolates to *S. cerevisiae*. The results, inarguably, give a true reflection of the composition of yeast strains involved in the alcoholic fermentation of sorghum wort for the production of "Dagarti *pito*" and thus repudiate the suggestion of some locality-dependent diversity in yeast populations involved in alcoholic fermentation of *pito* in Ghana. It is worth stressing that the other yeast species reported to be co-existing with *S. cerevisiae,* belong to genera included among the traditional non-*Saccharomyces* 'wild yeasts' which have been found to be brewing contaminants, causing various types of beer spoilage such as production of film, haze, off-flavors and superattenuation (Ingledew and Casey, 1982; Campbell and Msongo, 1991; Campbell, 1996 and Jespersen and Jakobsen, 1996). The two (1%) *Candida kefyr* isolates from one of the sites—Suhum (SH) – could have been contaminants and therefore irrelevant.

According to Vaughan-Martini and Martini (1998), to be identified as *S. cerevisiae,* an isolate must be able to assimilate glucose, sucrose, maltose, trehalose, raffinose and ethanol. One hundred and seventy-nine (72%) out of the 247 yeast isolates

showed macromorphological and micromorphological characteristics as well as carbohydrate assimilation profiles typical of *S. cerevisiae* and were easily identified with the API ID 32 C Kit as such. The remaining 68 (27%) isolates with similar macromorphological and micromorphological characteristics could not be identified in API galleries (Table 4.3) as *S. cerevisiae* because they possessed carbohydrate assimilation profiles that were not in full agreement with the taxonomic key for identification of yeasts particularly members of the *sensu stricto* complex as prescribed by Vaughan-Martini and Martini (1998). Whereas 61 out of these could assimilate glucose and maltose as carbon sources, 5 could only assimilate glucose with the remaining 2 assimilating glucose in combination with either melezitose or melezitose and palatinose. None of them could assimilate sucrose, trehalose, raffinose or ethanol. They however qualified to be considered as strains of *S. cerevisiae* in accordance with the description given by Barnett *et al.* (2000). The occurrence of *S. cerevisiae* strains with narrow carbohydrate assimilation spectra seems to be peculiar to *pito* yeast over a wide area of Ghana, having been reported earlier by Demuyakor and Ohta (1991), van der Aa Kuhle *et al*. (2001) and Naumova *et al*. (2003).

The observed exclusiveness of 39 carbohydrate assimilation profiles to 8 sampling sites Accra (13 profiles); Ayigya-Kumasi (8 profiles); Cape Coast (3 profiles); Tamale (4 profiles); Takoradi (4 profiles); Monaco-Kumasi (3 profiles) and Sunyani (3 profiles) and Ho (1 profile) suggests the indigenization of certain strains (Table 4.4). This observation rather shows that the composition of yeast

populations responsible for alcoholic fermentation of *pito* is not dependent upon the location of the production site and re-affirms the contention of van der Aa Kuhle *et al*. (2001) that reported variations could be due to deviations in the methods of isolation and characterization used.

Amplification of the region spanning ITS-5.8rDNA-ITS2, followed by restriction with *Hae*III indicated a close semblance of the isolates with atypical assimilation profiles to those readily identified in API galleries as *S. cerevisiae*, both possessing similar restriction profiles of four bands ranging in size between 115 and 316 bp (Table 4.5) and thus implying that they belonged to this species. Since identical restriction profiles were obtained for phenotypically different isolates, it could be concluded that ITS-PCR +RFLP is unsuitable for discriminating at subspecies level. This is in accordance with studies by several authors in which the method was found useful for differentiation of yeasts at species level, but inadequate for distinguishing between microorganisms at subspecies level (Guillamón *et al*., 1998; McCullough *et al*., 1998; Esteve-Zarzoso *et al*., 1999 and Pramatefki *et al*., 2000).

The largest amplified product size of 875 base pairs for the first group of five isolates is, however, at variance with the 880 base pairs reported for *S. cerevisiae* type strain CBS1171 and African sorghum beer yeasts (Las Heras-Vazquez *et al*., 2003, Naumova *et al*., 2003). Valente *et al*. (1996) reported of *Saccharomyces* strains from various origins with ITS products amplified by primers ITS1 and ITS4 ranging in size from 690 to 860 bp.

According to Towner and Cockayne (1993), conventional agarose gel electrophoresis methods are incapable of separating DNA molecules greater than 50-100kb in size, while microbial chromosomes are typically several megabases in size. One way to overcome this problem of size is to digest the chromosomal DNA with a restriction endonuclease to yield DNA fragments that are capable of resolution by standard electrophoresis methods. Restriction endonucleases are enzymes that recognize a specific base sequence of DNA, typically 4-6 base pairs (bp) long, and then cleave the DNA at a defined position in relation to the specific recognition sequence. The specificity of DNA cleavage by these enzymes means that complete digestion of a particular sequence of DNA by a specified enzyme or combination of enzymes will result in the production of a reproducible set of linear fragments, generated according to the frequency and location of the specific enzyme recognition sequence(s). Three isolates—A13, CC8 and SH2 did not show any restriction fragments. Perhaps no PCR products were available for digestion at all or too few fragments were produced during restriction analysis which were difficult to electrophorese by conventional techniques because of their size. T15, T16, T35, N3 and N4 showed chromosomal DNAs (PCR products) that were not digested during restriction. Lack of digestion of the PCR products could have resulted from the inability of the

Hae III restriction endonuclease used to recognize specific base pair sequences on the DNA molecules of those isolates in order to cleave them at defined positions to produce a 'fingerprint' of the linear DNA restriction fragments following

electrophoresis. Isolates N22 and T14 possessed additional restriction fragments. Whereas it is normal for so many fragments to be produced during restriction analysis, the additional bands present in N22 and T14 could be caused by the presence of extrachromosomal plasmid or bacteriophage DNA as *Hae* III endonuclease has been found to cleave the PCR products (DNAs) of members of the *Saccharomyces* sensu stricto complex into three or four bands only during restriction (Esteve-Zarzoso *et al.*, 1999; Naumova *et al.*, 2003). It is imperative, therefore, to first analyze undigested DNA by electrophoresis on agarose gels to test for the presence of any bands additional to that of the chromosomal DNA.

Demonstration of chromosome length polymorphism (CLP) during PFGE of isolates with phenotypic characteristics typical and atypical of *S. cerevisiae* has shown that they all belong to *S. cerevisiae* by possessing polymorphic chromosome profiles with band sizes between 200 and 1900 kbp (Fig. 4.3). A similar finding of chromosome length polymorphism was reported by van der Aa Kühle *et al.*, (2001) in yeast isolates from West African sorghum beer from Northern Ghana and Burkina Faso. This distinct variation is a strong indication that *pito* fermentation involves a large number of strains of *S. cerevisiae*.

Whereas in most of the isolates, chromosomes V and VIII were separated or almost separated as for *S. cerevisiae* type strain CBS 1171, some isolates showed chromosome length polymorphism both by size and number of bands. Pulsed Field Gel Electrophoresis (PFGE) analysis of genomes digested with 'rare-cutting' enzymes has been found to overcome the problem of restriction endonuclease

analysis yielding a complex pattern of poorly resolved fragments. The pattern of restriction fragments is characteristic of each strain and provides an estimate of the degree of genomic relationship between strains. Thus, closely related strains that differ by only a few bands can be identified readily by side-to side visual comparison of the fingerprint patterns on the same gel (Towner and Cockayne, 1993). Even though laborious, PFGE is inarguably a suitable method for subspecies typing as earlier observed (Hayford and Jespersen, 1999; Jespersen *et al*, 1999; van der Aa Kühle *et al*., 2001).

The close clustering of isolates of varying physiological characteristics from same and different sampling sites also gives strong indication that they are conspecific and belong to *S. cerevisiae* (Fig.4.4). The merging of the two large clusters at 73% similarity gives a very high degree of resemblance between the isolates, irrespective of their physiological differences and origin. By merging with the smaller clusters which consist of fewer isolates at 50-60% similarity, the members of the large clusters can be said to be closely related to those isolates as well.

Sequencing of the mitochondrial gene *COX2* of representative isolates followed by alignment with the known sequences of the *Saccharomyces sensu stricto* spp particularly *S. cerevisiae* type strain CBS1171 showed an average alignment similarity of 98.7% nucleotides (Table 4.6), suggesting their close-relatedness to *S. cerevisiae* (Vaughan-Martini and Martini, 1998). The homology difference of 1.3% (equivalent to about 8 nucleotides), however, seems to negate this suggestion. Earlier studies by Kurtzman and Robnett (2003) found a large number of

nucleotide substitutions in the *COX2* gene among strains of *S. cerevisiae, S. kudriavzevii and S. mikatae* while Fischer *et al.* (2000) also showed diversity in the *COX2* gene of *S. kudriavzevii* mating types. A high homology difference of 3.4% encountered by van der Aa Kühle and Jespersen (2003) between *S. boulardii* and *S. cerevisiae* type strain CBS1171 has been attributed to inter-strain variations. The present findings are therefore consistent with general observations that *COX2* gene sequences may exhibit diversity even within closely related strains of a species (Kurtzman and Robnett, 2003).

No significant change was observed in pH during the fermentation of *pito* and the final product had an average pH of 3.49 (Table 4.7). *Pito* is known to be slightly sweet to sour and contains lactic acid (Ekundayo, 1969; Bansah, 1990). This mild acidity is known to be advantageous in preventing growth of spoilage bacteria and moulds, thus enhancing the shelf life of the product.

There was no apparent correlation between taxonomic characteristics of isolates and production sites from which they originated. Even though there was no correlation between growth patterns and the locality of isolates, the carbohydrate assimilation spectra of some isolates seemed to have largely influenced their growth. Thus some isolates, which could assimilate only two carbohydrates, displayed minimal cell multiplication throughout fermentation, in contrast to those with broad assimilation spectra, which exhibited exponential growth (Fig. 4.5). The decrease in growth rate of some isolates after some time might have been due to competition or other unfavorable growth conditions during fermentation.

Majority (90%) of the isolates investigated for their ability to impart sensorial properties such as aroma, taste and mouth feel gave products of comparable organoleptic quality to the commercially produced "Dagarti *pito*" in terms of one or the other property but not all three (Fig. 4.6). For instance *pito* produced with one isolate, while having mouth feel very similar to that of the commercial product, tasted only quite similar to it and had a different aroma from commercial pito. The inability of any individual isolate to produce *pito* having mouth feel, taste and aroma very similar to the commercial product as evaluated by the panelists seems to imply that only various combinations of these isolates could yield *pito* with the desired attributes. Similar studies by Demuyakor and Ohta (1993) using single cultures of *S. cerevisiae* and crude mixed yeast cultures revealed that the mixed yeast culture gave an aroma typical of *pito*, while the single culture of *S. cerevisiae* produced an atypical *pito* with a dry and slightly bitter taste. Perhaps the ability of each isolate to produce metabolites that could contribute to sensorial quality is positively enhanced by the synergistic activities of one or more isolates present in a mixed culture, thus ensuring better organoleptic quality of the product.

Seventeen out of the nineteen isolates investigated did not exhibit flocculation while two were flocculent with sediment volumes of 0.75 ± 0.21 and 1.4 ± 0.00, respectively after 10 minutes (Table 4.9). The flocculence of yeasts is generally regarded as of importance in brewing. The cells of flocculent yeasts, by their aggregation into more or less large clusters, have limited access to the wort than those of non-flocculent yeasts in which clustering is absent; flocculent yeasts

therefore ferment less vigorously, and where they are bottom yeast, sediment out of the beer so rapidly, thus terminating the fermentation before the consumption of available fermentable sugars (Speers *et al.,* 1992). Flocculent top yeasts differ from bottom yeasts in rising to the surface instead of settling at the bottom of the fermenting wort, but the final effect on the beer is the same. The two flocculent isolates observed in this study, which came from different sites, also differed in their flocculation patterns, one sedimenting at the bottom (bottom-fermentor) and the other forming clumps at the surface of the suspension (top-fermentor). It may be concluded that flocculent yeasts tend to produce a less turbid and less completely attenuated beer at racking than non-flocculent yeasts. The non-flocculent nature of majority of the *pito* yeast isolates makes them better fermentors and therefore useful in brewing *pito*. Flocculence behavior of *pito* yeast has never been reported. The observation of the two flocculent isolates could probably be a new finding (Fig. 4.7).

While cell flocculation has been examined for over a century and has been the subject to a number of reviews in the early part of this decade, our view of the process is cloudy. Flocculation is affected by cell genetic behaviour, cell age as well as the chemical and physical nature of the surrounding medium. Recently, a number of advances in our understanding of the genes governing the process have occurred, in conjunction with the development of new assay methods (Jin and Speers, 1998).

The ten isolates from Ghana investigated for aroma production capability during fermentation of *pito* wort variously produced several aroma compounds belonging to the general groups of alcohols, esters, ketones, and aldehydes (Table 4.10). These are among reported typical flavor compounds of conventional beer (Lewis and Young, 1995). Annan *et al*. (2003b) reported that fermented maize dough made from *S. cerevisiae* contained several esters, fusel alcohols (such as 1-propanol, 2-methyl-1-propanol and 3-methyl-butanol), and ethyl acetate. In their studies, Damiani *et al*. (1996) found the fusel alcohols, 2-methyl-1-propanol and 2/3-methyl-1-butanol, with their respective aldehydes and ethyl acetate, were characteristic volatile compounds of sourdough started with fermentative yeasts belonging to the genera *Saccharomyces* and *Hansenula*. The isolates from Burkina Faso did not possess strong ability in producing volatile compounds as compared to the Ghanaian isolates.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

A total of 249 isolates have been obtained during a survey of "Dagarti *pito*" production sites from ten localities in eight geographical regions of Ghana. Phenotypically, the isolates showed differences in carbohydrate assimilation. Fifty representative lots of two hundred and forty-seven (247) of these have, however,

been proved using several molecular biology techniques to be strains of *S. cerevisiae*.

Twenty-five representative strains have been found to possess desirable technological properties, including sufficient growth during fermentation and efficient hydrolysis of sugars for biomass enhancement and fermentation activities, particularly, ethanol production, formation of aroma compounds and metabolites, which impart appropriate sensory attributes to *pito*.

This indicates a diversity of *S. cerevisiae* strains involved in fermentation of *pito* wort. Selection and development of starter cultures from this large population and use across the country will produce the same kind of "Dagarti *pito*", thereby making it a commercially viable product.

Another implication is that Ghanaians anywhere in the world can prepare and enjoy home-brewed *pito* if they get access to the starter cultures to be developed.