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Isolation and Identification Of Yeast Associated With Fermented Orange Juice

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----------ABSTRACT----------
Yeast species present in orange juice were screened. A total of 98 strains of yeast were isolated from fresh or healthy fermented and defective orange fruit. A total of 51 species were identified using AP120C, representing 4 genera; 32 from fresh fermented orange juice (FSSOJ) which 19 species were from defective orange juice (DSSOJ). Among (FSSOJ) isolates, Candida kruesi and Rhodotorula minuta were the predominant species, while Candida zeylanoides and Candida parapsilosis were the dominant species in DSSOJ. Candida and Rhodotorula species were both isolated from fresh or healthy and defective orange juice, while Kodamaea and Geotrichum species were isolated from the fresh or healthy orange juice only. Candida Kruesi had the highest prevalence (57%), Rhodotorula minuta (20%), Candida zeylanoides (8%), Candida parapsilosis (6%), Geotrichum capitatum (4%), Candida norvegensis and Kodamea (1%) respectively. Candida lusitaniae, Candida parapsilosis and Rhodotorula minuta metabolised xylose which showed that they possess genes responsible for xylose fermentation. Thus, these make them suitable for ethanolic fermentation of hydrolysates of cellulosic materials. The diversity of yeast isolated from the FSSOJ and DSSOJ showed that orange juice employ a whole range of natural flora that could function under varied environmental conditions.

***KEYWORDS**: Yeast, Candida species, Kodamaea ohmeri, orange juice, FSSOJ, DSSOJ*

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I. INTRODUCTION

Orange (*Citrus sinensis*) is one of the largest citrus grown fruit in Nigeria (Sumati, 1977) and also one of the most economically important tree crop in the world (Spiegel-Roy and Goldschmidt, 1996). The majority of citrus arrives the market in form of processed products, such as single-strength orange juice and frozen juice concentrates. In Florida, one of the world's top producing regions, more than 96% of all oranges are processed into orange juice. In the 1999-2000 seasons, Florida produced more than four billion litres of single-strength orange juice (Anon, 2001). India produces about 9 million tons of fruits every year growing at a rate of 12% per annum. The total market potential for fruit juices is 230 million litres including both packed and freshly made fruit juices (Tambekar *et al.*, 2009, Keshari, 2010,). From reports (Guillotin *et al*., 2009; Chen *et al*., 2010; Petrisor *et al*., 2010), fruit undergo tremendous chemical changes once separated from the parent plant, until finally spoilage sets in as a result of attack from bacteria, yeasts and fungi. Typical changes may show in texture, colour, flavor and respiratory activity which affects the processed fruit juices.

Apart from microbial invasion of plant tissues during various stages of fruit developments, a second factor contributing to microbial contamination of fruits (oranges, lemons, apple) pertains to their post harvest handling (Fernandez-Trujillo, *et al*., 2009) and through enzyme preparation for food processing (Fernandes, 2010).Pathogenic organisms can enter fruits and vegetables through damaged surfaces, such as punctures, wounds, cuts and split. This damage can occur during maturation or during harvesting and processing (Badge and Tumane, 2011). Between 1974 and 2012, numerous illness outbreak associated with unpasteurized fruit juice and cider have been reported world wide, involving approximately 2,527 cases (Mihajlovic *et al*., 2013). Ten out of these outbreaks were associated with orange juice contaminated with *Enterotoxigenic E. coli*, *Salmonella* spp, *Shigella* spp and *Hepatitis A.* (Mihajlovic *et al*., 2013).Yeasts are actually microbial eukaryotes which belong to ascomycetes that are good source of vitamin B and protein. Yeast are plant-like unicellular fungi thriving on every living organism. Being living organism fungi require warmth, water, albumen or nitrogenous material and sugars to remain alive (The Artisan, The Yeast Treatise, 2002). Yeasts thrive in habitats, where sugars are present, such as fruits, flowers and bark of trees. However, saleable yeasts of today are fairly different from wild strains due to genetic treatment, allowing them to grow in inappropriate situations (Madigan *et al*., 2003).

Citrus juices are acidic beverages with sugar content (15^0 Brix). Under these conditions, acidolactic bacteria, moulds and yeast comprise the typical microbiota. Typical yeast species found in citrus juices are *Candida parapsilopsis, Candida stellata, Candida intermedia, Saccharomyces crataegensis, Saccharomyces cerevisiae, Torulaspora delbrukii and Zygosaccharomyces ruoxii.* Also included are species from the genus *Rhodotorula Pichia, Hansenispora and Metschikowia* (Hatcher, *et al*., 2000; Covandonga *et al*., 2002).
Yeast species are used in many industrial fermentation processes including alcohol beverages production and leavening or rising of bread dough. Despite the economic importance of citrus juices, there are few reports investigating yeast species associated with them (Parish and Haggins, 1989; Deak and Beuchat, 1992 and Hatcher, et al., 2000). Therefore, detailed study of these yeast isolates from citrus juice is needed, thus, this study was carried out to exploit the diversity of yeast that could be found in orange juice.

II. MATERIALS AND METHODS

Sample collection and preparation : One hundred and fifty (150) medium sized mature ripe sweet oranges healthy and defective fruits (soft rot fruits, damaged surfaces such as wounds) were purchased from a local market in Zaria, Kaduna state. They were thoroughly washed and surface decontaminated by placing the whole fruits in 80^0C water bath for two minutes. The oranges were then peeled, sliced and juice extracted using the stainless steel blender. The juice was clarified or filtered by the use of muslin cloth (sterile) into sterile bottles under aseptic condition. The samples of the healthy or fresh single-strength orange juice (FSSOJ) were stored for two and seven days interval at room temperature (26-28^0C) before analysis. The defective fruits single-strength orange juice (DSSOJ) was analysed immediately after the extraction of the juice. The processing of juice and analysis was done in the Department of Microbiology Laboratory, Ahmadu Bello University, Zaria.

Isolation of the yeast strains : The yeast strains were isolated from the fresh and defective orange juice (FSSOJ and DSSOJ) using the techniques of Beech and Davenport (1971) with some modification. Serial dilution of each of the various samples (FSSOJ and DSSOJ) was made. The stock culture was prepared by taking 50 ml of the orange juice samples and mixed with 450 ml of sterile peptone water. Each sample was serially diluted (10 fold dilution of 1:10, 1:100, 1:1000, 1:10,000) in sterile peptone water. An aliquot of 0.1ml of each sample was plated out on potato dextrose agar (PDA) (Oxoid) and incubated at ambient temperature (26-28^0C) for 48-72 hours.

Maintenance of culture : The discrete isolated colonies (pure cultures) was picked out purified by re-streaking on PDA plate and maintained on slants of the same medium at 5^0C in the refrigerator.

Characterization of yeast strains : The yeast strains were obtained based on their morphological and physiological characteristics and were identified to species level using AP120C AUX KIT (bioMèriux, Lyon, France) according to the method described by Okafor, (1971), Espinel *et al*., (1998) and Moghaddas, *et al*., (1999).

The Morphological and physiological test include: colonial and vegetative morphology, Pseudomycelium, pellicle formation, incubation at different temperatures of 30, 35, 37 and 42^0c. Glucose utilization at 50% (w/v), sodium chloride at 3 and 10%(w/v).

Carbon assimilation test : The carbon assimilation activities of the yeast species on carbohydrates were carried out using the AP120C AUX strips which comprised of 19 assimilation tests of (D-glucose, glycerol, 2-keto-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, Inositol, D-Sorbitol, Methyl-α D-glucopyranodsides, N-acetyl-glucosamine, D-Celloboise, D-trehalose, D-Melezitose and D-raffinose respectively.

III. RESULTS

A total of 51 yeast isolates were obtained based on their morphological and physiological characteristics and were identified to the species level using AP120C AUX KIT (bioMèriux) from the samples of fresh and defective single -strength orange juice (FSSOJ and DSSOJ).The result of the morphological and vegetative characteristics showed that the colonies of the yeast strains isolated were spherical, creamy smooth, raised and flat, some appeared pinkish, smooth, shiny and flat. Microscopically, the vegetative cells are spherical, oval, elongated with single or multipolar budding (Table 1). Physiological properties such as growth on 50%(w/v) glucose, ethanol (3% (w/v), sodium chloride 3% and 10% w/v and growth at elevated temperatures showed that all the yeast species isolated grew at the various elevated temperatures of 30,35,37 and 42^0C. All the yeast strains utilized glucose except *Geotrichum* species and grew at 3% sodium chloride, but

some species of *Candida* and *Geotrichum* did not grow at 10% (w/v) sodium chloride (Table 1). The prevalence of the yeasts isolated and identified showed that *Candida Kruesi* 29(56.86%) had the highest isolation rate followed by *Rhodotorula minuta*, 10 (19.60%). Others incude *Candida zeylanoides* 4(7.84%), *Candida parapsilosis* 3(5.88%), *Geotrichum capitatum* 2(3.92%), *Candida lusitaniae* 1(1.96%), *Candida norvegensis* 1(1.96%) and *Kodamea ohmer i* 1(1.96%) (Fig.1).

Table 1: Physiological characteristics of yeast strains isolated from fresh and spoilt single-strength orange juice.

PTM	PF	GG	GE	GN_1	GN_2	30^{0C}	35^{0C}	37^{0C}	42^{0C}	Suspected Organism
+/-	+/-	+	+	+	+/-	+	+	+	+	*Candida spp*
+/-	-	-	+	+	-	+	+	+	+	*Geotrichum spp*
-	-	+	+	+	-	+	+	+	+	*Kodamaea spp*
-	+/-	+	+	+	+	+	+	+	+	*Rhodotorula spp.*

Key: **PTM** =Pseudomycelium/true mycelium **PF** = Pellicle formation
GG = Growth on 50%w/v glucose **GE** =Growth on 3% (v/v) ethanol
GN_1 =Growth on 3% (w/v)NaCl **GN_2**=Growth on 10% (w/v) NaCl

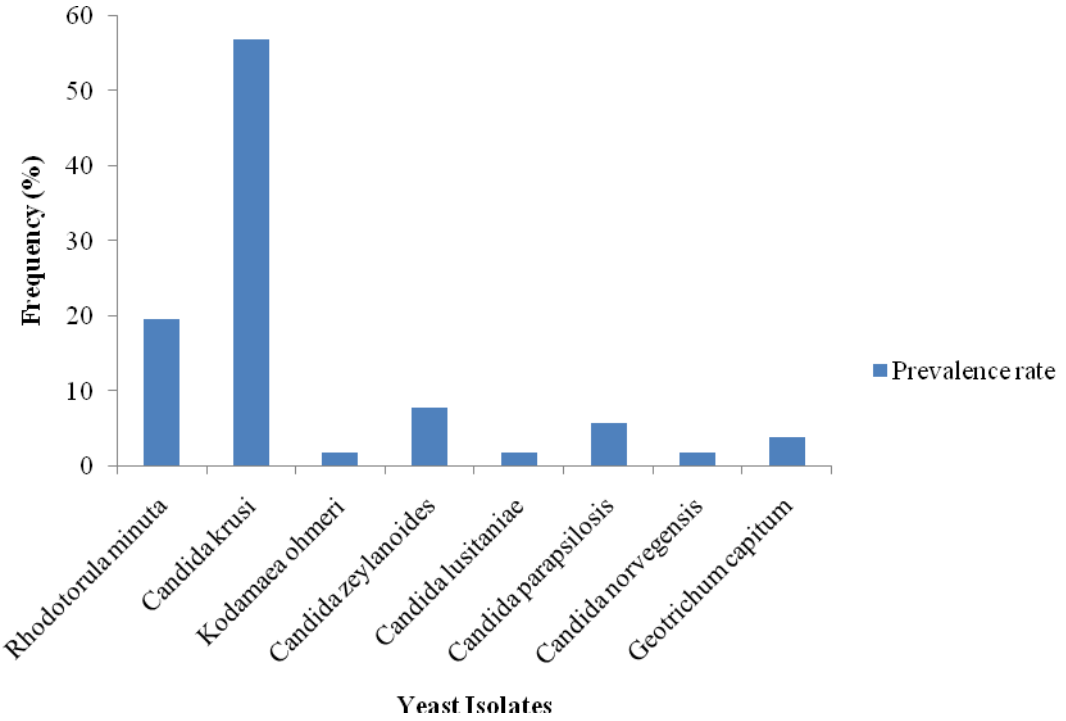

Fig 1: Prevalence rate of yeast strains isolated from fresh and spoilt single strength orange juice

Carbon assimilation test

The result in Table 2 showed that the yeast strains of *Candida lusitaniae, Candida parapsilosis, Kodamea ohmeri* and *Rhodotorula minuta* assimilated glycerol, calcium 2-Keto-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, Sorbitol, Methyl-α D-glycopyranoside, N-acetyl-glucosamine, D-maltose, D-sucrose, D-trehalose and D-melezitose respectively. Candida zeylanoides assimilated glycerol, Calcium 2-Keto-gluconate and N-acetyl-glucosamine. *Candida kruesi* assimilated only glycerol and N-acetyl-glycosamine. *Geotrichum* species did not assimilate any of the sugars tested but produced pseudohypae, while *Candida norvegensis* assimilated only D-glucose as the sole carbon source.

Table 2: Carbon assimilation test using API20C AUX Kit (bioMèriux)
Key: + = Carbon assimulated (turbid), - = Carbon not assimulated (non-turbid), ± =variation

GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	IDENTITY
+	+/-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	***Candida krusei***
+	+	+	-	+	+	+	+	-	-	+	+	-	-	+	+	+	+	-	***Candida lusitaniae***
+	+	+	-	+	+	+	+	-	-	+	+	-	-	+	+	+	+	-	***Candida parapsilosis***
+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	***Candida zeylanoides***
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***Geotrichum capitatum***
+	+	+	-	-	+		+	-	-	+	+	-	-	+	+	+	-	-	***Kodamaea ohmeri***
+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	+	+/-	+/-	-	***Rhodotorula minuta***
+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***Candida norvegensis***

GLU = D-glucose ADO =adonitol MDG =methyl-α-D-glucopyranoside SAC =D-sucrose
GLY = glycerol XLT = xylitol NAG =N-acetyl-glucosamine TRE = D-trehalose
2KG = calcium 2-keto-gluconate GAL = D-galactose CEL = D-cellobiose
ARA = L-arabinose INO = inositol LAC =D-lactose
XYL = D-xylose
SOR =sorbitol
MAL =D-maltose MLZ = D-melezitose RAF= D-raffinose

IV. DISCUSSION

Morphological and physiological characteristics of yeast strains isolated from FSSOJ and DSSOJ

Yeasts capable of growing on solid surfaces tend to form colonies with distinctive morphology, this is because individual species often form colonies of characteristics size and appearance as reported by (Prescott et al., 2008). This statement agrees with the result obtained in this study since all the yeast strains isolated were similar in colonial appearance and vegetative morphology that shared the same oval, spherical shape and budding as a means of asexual reproduction,The diversity of yeast isolated from the fresh and defective single-strength orange juice utilized for this work showed that most citrus juices employ the whole range of natural flora that could function under the varied environmental conditions, which can affect the vitality and viability of the cultures as reported by (James et al., 2003). Thus, the result of this study revealed that *Candida* species were more in number. *Candida kruesi* had the highest prevalence of (56.86%), *Rhodotorula minuta* (19.6%), *Candida zeylanoides* (7.84%), *Candida parapsilosis* (5.88%), *Geotrichum capitatum* (3.92%), *Candida lusitaniae*, *Candida norvegensis* and *Kodamea ohmeri* (1.96%) respectively (Fig. 1). The yeast strains obtained in this study were able to grow at elevated temperatures slightly higher than room temperature (Table 1). The survival

of all the wild yeast strains obtained in this study grown at physiological temperatures (room temperature) and then subjected to mild heat shock (35-37^{0}C) and lethal heat shock (42^{0}C) agrees with the report of Piper, (1993) that prior induction of the heat shock responses enables cells to survive subsequent exposure to lethal high temperatures.

Carbon assimilation profile : Carbon assimilation is an important criterion in the taxonomy and identification of yeast which depends on organic carbon sources for their energy supply and growth (Table 2). Galactose is a non-conventional nutrient for yeasts, which however can be used for as a sole carbon source when glucose is absent from the medium. Thus, the ability of yeast strains of *Kodamea ohmeri*, *Candida lusitaniae* and *Candida parapsilosis* isolated in this study to assimilate galactose indicated that the strains have the GAL genes responsible for galactose fermentation (Yun *et al*., 2001) (Table 1). Furthermore, the ability of these isolates to also ferment maltose shows that they possess uptake mechanism that involves two systems; an energy – dependent maltose permase (ATP $\leftrightarrow$ ADP) which transports the maltose intact across the cellular membrane and a maltose (alpha – glucosidase) which hydrolyses maltose internally to yield two glucose units (Stewart, 2006). Thus, the mechanism is mediated genetically by three maltose utilisation genes (MAL genes) which are involved in the operation of the high-affinity maltose transporter (Lodolo *et al*., 2008) (Table 2).The ability of our isolate *Candida lusitaniane*, *Candida parapsilosis* and *Rhodotorula minuta* to metabolise xylose shows that the strains possess xylose reductase and xylitol hydrogenase genes responsible for xylose fermentation (Kosman, 2003) (Table 2). Thus, these make them suitable for ethanolic fermentation of hydrolysates of cellulosic materials. All of the yeast strains obtained in this work metabolised glycerol except *Candida norvegensis*. This indicated that these strains possess the glycerol kinase gene (GuT1) and a gene for mitochondrial glycerol 3-phosphate dehydrogenase (GuT2) responsible for glycerol assimilation during fermentative growth (Palik *et al*., 1993; Ronnow and Kielland – Brandt, 1993) (Table **2).**An unconventional storage dissacharide found in yeast is trehalose (α,α – 1, 1 – diglucose) which exist in high concentration in resting and stressed cells. The yeast strains of *Candida lusitaniae, Candida parapsilosis, Kodamea ohmeri* and *Rhodotorula minuta* utilised trehalose and this showed that they have the gene responsible for synthesis of trehalose enzyme because the breakdown of trehalose to glucose is mediated by trehalose enzyme and both synthesis and degradation are regulated via cAMP (Versele and Thevelein, 2001). *Candida kruesi* and *Candida norvegensis* did not assimilate trehalose and this indicated that they lack this gene (Table 2). Summarily, it is well established that most of the yeast isolated employed different sugars as their main carbon and hence energy source.

V. CONCLUSION

The abundance and availability of the wild yeasts obtained in this study satisfy one of the basic requirements for use of microorganisms in fermentation industry. This is because most traditional fermentations such as fermented juices are characterized by numerous natural micro floras of yeasts of varying functions that could be beneficial or detrimental to the fermentation processes; mixed cultures that produce the blend of rich flavours and aromas of the product and some microorganisms that could accelerate spoilage, particularly in the finished products. Therefore, fermented products should be hygienically and pathogen-free for human consumption.

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