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Full Length Research Paper

Isolation and Identification of Local Ethanolic Yeasts Inhabiting Coffee Processing Environments in Tanzania

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Abstract

Coffee processing environment harbours different microbiota of fungi and bacteria, some of which are of great economic value. This study isolated, screened and identified indigenous yeast associated with ethanol production potential in coffee processing environments of Mbinga in Ruvuma region and Hai in Kilimanjaro region using standard procedures. Yeast identification was done by amplification and sequencing D1/D2 domain of the gene 26S rDNA. A total of 21 yeast isolates were obtained from both sites and only 8 of them were able to ferment glucose. All 9 isolates fermented coffee pulp waste to ethanol at varying levels. Highest ethanol production was observed by isolate M4, which produced 3% (v/v) ethanol, followed by PDA4MB (2.8% v/v), PDA3 (2.6% v/v) and the least was by M2 which produced only 1.1% (v/v).Three isolates showed good attribute to ethanol production (2.76 to 3.56% v/v) even after an addition of higher sugar concentrations to coffee pulp waste. The basic local alignment search tool (BLAST) and phylogenetic analysis revealed the identity of the yeasts as Pichia kudriavzevii, Issatchenkia orientalis, Clavispora lusitaniae, Pichia guilliermondii, and Pichia anomala, all of them known to be associated with ethanol fermentation. Their attributes towards ethanol fermentation are taken to be potential for further investigation for bioethanol production.

Key words: *Coffee pulp waste, Isolation, Ethanol, Yeast, Sequencing*

Introduction

Yeasts are for a long time known to be economically important in the biotechnology of fermented foods and beverages (Esteve-Zarzoso *et al*., 1999) and are classified into two phylogenetic groups: teleomorphic and anamorphic ascomycetous yeasts and teleomorphic and anamorphic basidiomycetous yeasts (Kurtzman and Fell, 1998). This economically significant fungal group has increased interest in applications of biotechnology (Sheela *et al.,* 2008), medical research (Rad *et al*., 2012) and biodiversity (Kurtzman and Robnett, 1998). They usually inhabit substrates with simple sugar, organic acids and easily utilized compounds (Glushakova *et al.,* 2007). The natural coffee fermentation process involves a diversity of yeasts (Silva *et al*., 2000) and filamentous fungi (Masoud and Kaltoft, 2006; Avallone *et al*., 2001). Several studies have isolated yeasts from natural fermentation of robusta coffee bean which includes *Kluyveromyces marxianus*, *Saccharomyces bayanus*, *Saccharomyces cerevisiae* var. *ellipsoideus* and *Schizosaccharomyces* spp in India (Agate and Bhat, 1966). In Brazil coffee fermentation environment, *Debaromyces hansenii* was found in highest amount (27%) followed by *Pichia guilliermondii*(18.9%), Candida spp (8%) and other yeasts such as *Pichia burtonii, Debaromyces polymorphus, Arxula adeninivorans, Pichia holstii* and *Pichia anomala* (Silva *et al.,* 2008a).

Molecular techniques are increasingly adopted for identification of fungi and yeasts over conventional techniques due to the fact that they are rapid, easy and more precise (Arroyo-Lopez *et al*., 2006). The 26S rDNA region has approximately 600 bases in size reported to be a strong tool which allows accurate identification (Daniel and Meyer, 2003) and has the advantage that yeast species can be identified from sequence divergence in this region (Kurtzman and Robnett 1998). Masoud *et al.* (2004) successfully identified yeasts involved in fermentation of coffee *arabica* using D1/D2 LSU of 26S rDNA. They included *Pichia anomala, Pichia ohmeri, Pichia kluyveri, Hanseniaspora uvarum, Candida pseudointermedia, Issatchenkia orientalis, Torulaspora del brueckii* and *Kluyveromyces marxianus*. Some of the mentioned yeasts have been reported to have pectinolytic activity (Masoud and Kaltoft, 2006, Agate and Bhat, 1966). Although earlier studies suggested that yeasts could be responsible for alcoholic taste in coffee beverage after over fermentation (Lopez *et al.,* 1989;Gibson and Butty, 1975), there is still a debate on the role of each microbe in fermentation (Avallone *et al*., 2001) and limited information is available on ethanolic yeasts involved in coffee processing and fermentation. In Tanzania, there is dearth of data on isolation and identification of yeast from Tanzanian coffee environments. Reported work in this direction so far is by Masoud and Kaltoft (2006) who identified fungi and yeasts with pectinolytic activity from Arusha coffee plantation and Ernest *et al*.(2014) who isolated citric acid producing fungi from coffee pulp residues.

This study focused on isolation and characterization of yeasts from the coffee environments in Tanzania able to ferment and convert coffee pulp waste into ethanol, using molecular methods based on sequence divergence on the D1/D2 region of 26S rDNA.

Material and Methods

Study sites

The study sites were Mbinga in Ruvuma and Hai in Kilimanjaro regions known for high production of coffee in Tanzania. In both regions *Coffee arabica* is mostly grown and the wet method is used to process coffee. Mbinga samples were collected from Mbiku coffee processing estate which is in Mbinga district. Coffee in Mbinga is grown in Matengo highlands with attitude of 1500-1900 m above sea level. In Kilimanjaro, collection was done from Lyamungo Coffee Estate which has 290 hectares of coffee cultivation, located in Hai district which is among the biggest coffee plantations in Tanzania.

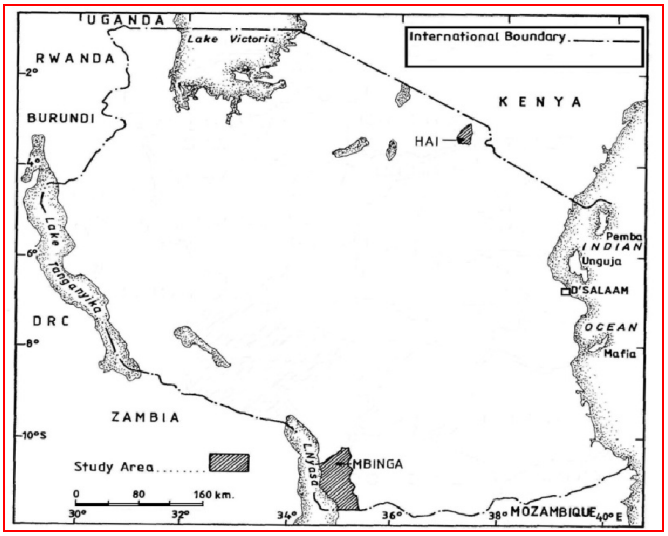

Figure 1: A map of Tanzania showing the study sites, Hai and Mbinga Districts

Sample collection

Samples namely natural fermenting juice of coffee beans, coffee pulp and ripen coffee berries were collected from each of the study areas and preserved in ice boxes, then transported to the Department of Molecular Biology and Biotechnology laboratory, University of Dar es Salaam, Tanzania for microbial studies. At the laboratory, 2-3 scoops of the collected samples using pre-sterilized spatula were inoculated into 3 test tubes containing 15 ml of malt extract broth (MEB) and to 3 plates of Malt Extract Agar (MEA). The same procedure was done when coffee pulp was used as substrate. Inoculated plates and test tubes were incubated (Memmert Incubator, Omnilabo, Netherlands)at temperature range of 28-30^{o}C. In order to obtain pure colonies, twelve hours overnight culture of the inoculated test tubes above were serially diluted at 10^{-1}, 10^{-2} and 10^{-3} in 1% peptone water (peptone 1g/L, sodium chloride 0.5g/L at pH 7.4) and were then inoculated to MEA platesas described by Silva *et al.* (2008b).The pure isolates were kept in a refrigerator at 4 ^{o}C for further studies.

Ethanol fermentation from glucose

All yeast isolates were first screened for their ability to ferment glucose, which is considered to be the simplest and most preferred sugar for ethanol production by yeast (D'Amore *et al.,* 1989). Two (2.0 ml) of yeast isolate was inoculated in 8.0 ml glucose media in test tubes containing inverted Durham tubes to measure carbon dioxide evolution (Trindade *et al.,* 1999; Van Dijken *et al.,* 1986). The fermentation mediaprepared as previously described by Brooks (2008), contained (w/v) glucose 2.0%, yeast extract 0.3% and peptone 0.5%. Any yeast that did not produce or produced very little carbondioxide was regarded as non-fermentative and those which produced from 50% and above were regarded as good fermentors (Melo *et al*., 2007).

Ethanol fermentation from Coffee pulp waste

All yeast isolates categorized as good glucose fermentors were further screened for their ability to ferment coffee pulp waste. Conical flasks of 250ml containing 100 ml of coffee pulp waste were sterilized and then added with 5 ml of twelve hours overnight culture of yeasts suspension and incubated at an ambient temperature (28-30^{o}C). Fermentation proceeded for four consecutive days. 1.5 ml of each sample were withdrawn daily and centrifuged (Mikro 220- Hettich machine: CH 8806 Bäch, Germany) at 13000 rpm for 3 minutes to get clear solution, then transferred to new eppendorf tubes. Ethanol analysis was carried out using gas chromatograph (Varian CP-3800, USA).

The ability of ethanol producing isolates to carry out fermentation in increased sugar concentrations was evaluated on three isolates by adding sugar to coffee pulp waste in a concentration of 5% (5 g of sugar per 100 ml of CPW) and 10% (10 g of sugar per 100 ml of CPW). Flasks were sterilized and then inoculated with the respective isolates and incubated at an ambient temperature (28-30^{o}C). After 96 hours, samples were centrifuged and the liquid part was taken for ethanol analysis.

Ethanol analysis

Ethanol analysis was performed according to the procedure followed by Hamadi *et al.*(2014) by using gas chromatograph (Varian model CP-3800, USA) with an auto injector and auto sampler system, equipped with flame ionization detector (FID)set at 280 ^{0}C and an injector at 250 ^{0}C with split state at ratio of 50. Separation was effected in a 30 m, 0.25 mm and 1 µm column type CP- SIL

8 CB, with the temperature maintained at 45- 55 ^{0}C at a rate 2 ^{0}C/min for 10 min then at 10 ^{0}C/min to 200 ^{0}C. Column flow was employed at 1.5ml/min with nitrogen as a carrier gas and hydrogen as a combustion gas at 20 psi; linear velocity was 39.0 cm/sec and a total flow of 116.2 ml/min. Samples were diluted with deionized water and to all samples and standards, butan-1-ol was added as internal standard at 0.25%.

Concentrations of ethanol were calculated by relating the peak areas of the sample and standards to their corresponding peak areas of the internal standards using the following formula:

Ethanol Concentration % (v/v) = $\frac{Ms/As \times W}{Mi/Ai}$

Where; Ms = peak area of a ethanol in a sample, As = peak area of internal standard in the sample, Mi = peak area ethanol in the standard, Ai = peak area of internal standard in the standard and W = concentration of ethanol in the standard.

Genomic DNA (gDNA) extraction

All isolates were inoculated into 50 ml of Malt Extract Broth (MEB) and incubated at 30^{o}C for twelve hours overnight. DNA was extracted using a protocol from De Barros et *al.* (1998) using the biomass obtained from twelve hours overnight culture in MEB. An extraction buffer (2% Triton X100, 1% SDS, 100 mMNaCl, 10 mMTris-pH 8, and 1 mM EDTA-pH 8) was used and ice cold isopropanol for gDNA recovery. The DNA pellet was suspended in TE (10mM Tris-HCl, 1 mM EDTA-pH 8) buffer then stored at –20^{o}C.Estimation of quantity of gDNA was done using standard protocol from Sambrook *et al.*(1989) using UV-visible light spectrophotometer (JENWAY- Genova, Bibby Scientific Ltd, Dunmow, Essex. CM6 3LB-UK) at 260 nm in the range between 50 - 100 ng.

Polymerase chain reaction (PCR)

The gene of interest (Figure 2) of the region D1/D2 domain of 26S rDNA was amplified using a PCR machine (Applied Biosystems, Veriti^{TM}- Model 9902, Singapore). The targeted region is known for being reproducible and highly variable at resolving species level differences (Kurtzman and Robnett, 1998).

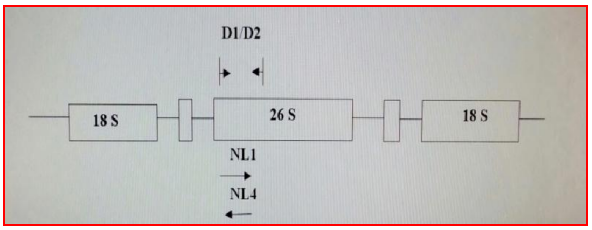

Figure 2: Schematic diagram of a ribosomal DNA (rDNA) showing primer binding site of the region D1/D2 of 26S rDNA of yeast. (Source: Sugita and Nishikawa, 2003)

The PCR reaction mixture contained 1µl of isolated gDNA, 22µl of sterilized distilled water, 1µl of each NL1 and NL4 primers. The final volume constituted of 0.1 mM of each dNTP, 1 × PCR reaction buffer, 2 mM $MgCl_2$, 1 U Taq DNA polymerase and 0.8 pmol/µlof each primer.D1/D2 region at the 5' end of 26S ribosomal DNA (rDNA) of about 600 base pairs was amplified with primers: NL1 5'GCATATCAATAAGCGGAGGAAAAG-3' (Forward) and NL4 5' GGTCCGTGTTTCAAGACGG-3' (Reverse) (Kurtzman and Robnett, 1998). The amplification process was carried out in36 PCR cycles, with denaturation temperature at 94 ^{o}C for 1 min, annealing at 52 ^{o}C for 1 min, extension at 72 ^{o}C for 2 min, and final extension at 72 ^{o}C for 7 min.

Sequencing and sequence analysis

PCR products were first purified using a QIANGEN kit using protocol given from the manufacturer (QIANGEN- QIAquick® PCR Purification Kit (50)- Sweden). The purified PCR products were then sent to INQABA, South Africa for sequencing. The procedure was done by the Big Dye chain termination method using an ABI 3730 Genetic analyzer. Each sample was sequenced twice, using either forward primer or reverse primer. The sequences were manually edited using *BioEdit v7.1.3* program then posted to NCBI website ***http://*blast.ncbi.nlm.nih.gov/** for sequence alignment (Altschul *et al*., 1997). Corresponding regions of other ascomycetous yeast of 26S rDNA were aligned with multiple sequences alignment by ClustalX program (Thompson *et al*., 1997) and phylogenetic tree was constructed by the neighbor-joining method in MEGA5 (Tamura *et al*., 2011).

Statistical analysis

All analyses were performed in triplicates and data was presented as mean standard deviation.

Results and Discussion

Yeast isolation and initial screening for fermentation ability

A total of 21 yeasts strains were isolated from the different samples collected from Mbinga (14 isolates) and Kilimanjaro (7 isolates) using MEB and MEA growth media (Table 1). Results on colony morphologies show that 12 strains had ovoid colonies and remaining 9 had spherical colonies. Majority of the colonies (15) were cream in color while 4 were pink and 2 were white colored.

Table 1: Characteristics of yeast isolates obtained from Mbinga and Kilimanjaro.

S/N	Isolate	Source	Shape	Colony color	Glucose fermentation capacity
		Samples from Mbinga			
1	MM1-1	Coffee berry	Ovoid	Cream	++
2	MM1-2	Coffee berry	Ovoid	Pink	+
3	MM1-5	Coffee berry	spherical	Cream	+++
4	MM2-1	Fermentation juice	spherical	Cream	++
5	MM4-1	Coffee pulp	spherical	Pink	+
6	MZ1-1	Coffee pulp	Ovoid	Cream	+ =
7	MZ1-2	Coffee pulp	ovoid	Cream	++
8	MZ1-3	Coffee pulp	spherical	Cream	++
9	MZ1-4	Coffee berry	ovoid	Cream	+
10	MZ1-5	Fermentation juice	ovoid	Cream	+
11	PDA2-MB	Fermentation juice	spherical	White	++
12	PDA3-MB	Fermentation juice	Spherical pherical	White	++
13	PDA4-MB	Fermentation juice	ovoid	Cream	+++
14	PDA5-MB	Fermentation juice	ovoid	Pink	+++
		Samples from Kilimanjaro			
15	M2	Coffee berry	Ovoid	Cream	+++
16	M4	Fermentation juice	ovoid	Cream	+++
17	M5	Coffee berry	spherical	Cream	+++
18	M6	Coffee berry	ovoid	Pinkish	++
19	PDA1	Fermentation juice	spherical	Cream	+++
20	PDA2	Fermentation juice	spherical	Cream	+++
21	PDA3	Fermentation juice	ovoid	Cream	+++

(+ = very weak (< 30% CO_2 gas in Durham tubes), ++ = weak (< 50% CO_2 gas in Durham tubes), +++ = good (≥ 50% CO_2 gas in Durham tubes)

In terms of glucose fermentation, only 3 isolates from Mbinga and 6 from Kilimanjaro showed good ability to ferment of glucose with ≥ 50% CO_2 gas in Durham tubes. The remaining were weak or non ethanolic yeasts. Majority of the glucose fermenters were cream in color, typical of yeast colonies. The result on fermentation of CPW by the selected best glucose fermenters revealed that M4 yeast isolate from Moshi produced highest amount of ethanol (3.0% v/v) followed by PDA4MB from Mbinga (2.8% v/v), PDA3 from Mbinga (2.6% v/v) and the least was M2from Kilimanjaro which produced only 1.1% (v/v)(Fig 3).

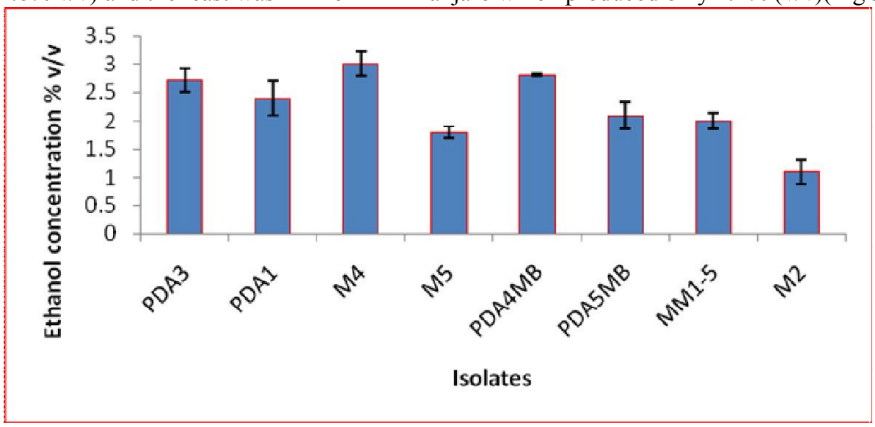

Figure 3: Ethanol production from CPW by yeast isolates. Values are means of three different measurements; Mean±SD, n=3. Error bars indicate standard error of the mean of the triplicates

Good physical fermentative attributes of yeast among others are alcohol production capacity, utilizing sugar at lower and higher concentrations and ability to convert sucrose to ethanol (Ali and Khan, 2014; Fadel *et al.,* 2013, and Nishida et al., 2004). When 3

best isolates selected on the basis of their ability to ferment coffee pulp waste were grown in CPW with added table sugar at 5% and 10%, M4 isolate produced highest amount of ethanol of 2.38% v/v and 3.56% v/vrespectively, followed by PDA4M and PDA3 (Figure 4). This however was a small increase when compared to ethanol produced from CPW without any addition of sugars. This small increase can be attributed to the fact that fermentation conditions were not optimized to enhance maximum ethanol production. These results are similar to those published by Fadel *et al.* (2013) who also reported 3.2-3.8% v/v ethanol during initial screening of yeast for ethanol production.

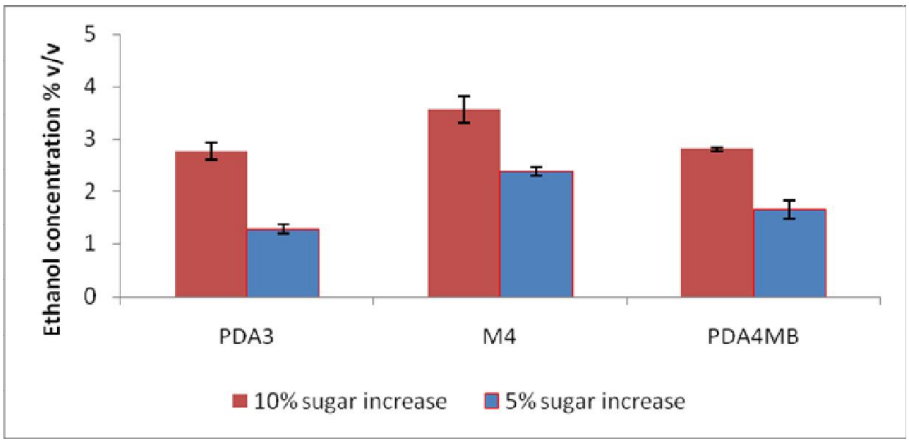

Figure 4: Ethanol production from CPW with table sugar at 5% and 10 %. Values are means of three different measurements; Mean±SD, n=3. Error bars indicate standard error of the mean of the triplicates.

PCR results

The approach used to identify yeasts was by PCR of 26S rDNA large sub-unit D1/D2, a region which has been reported to be variable and has ability to resolve individually defined species (Peterson and Kurtzman,1991), using D2 region, and by Kurtzman and Robnett (1998) using D1 and D2 LSU region of ascomycetes yeasts. In this study, the sizes of PCR productsof all isolates ranged from 550-600 bp (Fig 5), within the expected range in line with previous results by Kurtzman and Robnett (1998).

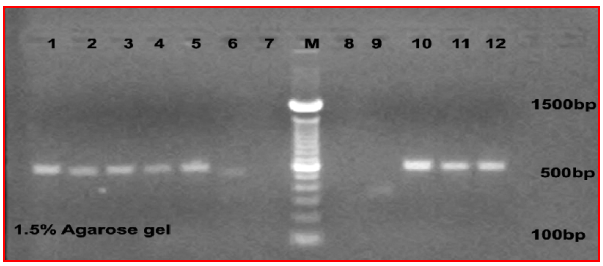

Figure 5: PCR products of yeasts using primers NL1/NL4 of the 26S LSU D1/D2
Key: Lane1: YPDA4MB, Lane2: PDA2, Lane3: YPDA5MB, Lane4: M2, Lane5: MM1-5, Lane 6: M5, Lane7: negative control, Lane M: 100bp marker, Lane8-9: empty, Lane10: M4, Lane11: PDA3 and Lane12: PDA1

Sequence alignment and phylogenitc relationships

Results from the sequence alignment *(http://*blast.ncbi.nlm.nih.gov/) revealed a pattern of similarities of yeasts isolated from this study to known ascomycetous yeasts from different sources (Table 2). Majority of the isolates (8 out of 9) perfectly matched known yeast strains in the NCBI database with similarity index between 99 and 100%. PDA2 matched with *Pichia kudriavzevii* by 99%, where as PDA 3 was perfectly identified (100% similarity) as *Issatchenkia orientalis.The* two yeasts have been isolated from coffee fermentation environment in Tanzania by Masoud *et al.*(2004). *Issatchenkia orientalis* was also isolated in the wine fermentation environment in Brazil (Baffi *et al*., 2011) and in Spain (Clemente-Jimenez *et al*., 2004) where it was reported to be vital for the production of higher alcohols (Clemente-Jimenez *et al.*, 2004). This might explain why PDA3 was among the best ethanol producer in this study. Both BLAST (Table 2) and Phylogenetic analyses (Figure 6) grouped together isolate *YPDA4MB* and *YPDA5MB*as *Meyerozyma guilliermondii* isolated from fermentation of Jerusalem artichoke.

In this study three isolates namely M2, MM1-5 and M2 had 99% similarity to *Clavispora lusitaniae* (*Candida lusitaniae*). This yeast has not been isolated from coffee environments in previous studies by Silva *et al.*(2008a); Masoud*et al.*(2004) and Avallone *et al.*(2001). Some researchers had suggested that this organism could be coupled in ethanolic fermentation with enzymatic hydrolysis at higher temperatures (Esteghlalian *et al.,* 2007; Kaar and Holtzapple, 2000).

Table 2: Identification of yeast isolates based on sequence alignment (BLAST)

Yeast Isolated	Nearest phylogenetic relative	Strain	Accession number	Source	Similarity (%)	Reference
PDA1	*Pichia anomala*	VTT C-04565	DQ 377650	Industrial malting	96	Laitila *et al.*(2006)
	Uncultured fungus	clone 28-239FM	HQ143112	barley malts	96	Kaur *et al.* (unpublished)Australia
PDA2	*Pichia kudriavzevii*	clone test65	JQ 419949	Sicilian-style fermented Sevillano olives	99	Golomb*et al.* (unpublished)-USA
	Pichia kudriavzevii	Y4	JF 715182	Mango-pulp compost	99	Dandi*et al.* (unpublished) India
PDA3	*Issatchenkia orientalis*	F701	EU 807918	marine yeast in East China Sea	100	Zheng et al. (unpublished)-China
	Issatchenkia orientalis	isolate 229	AY 529504	West African cocoa beans	99	Jespersen *et al.* (2005)
M2	*Clavisporalusitaniae*	LZ-5	JQ 686919	Jerusalem artichoke	99	Hu *et al.* (unpublished)-china
	Clavispora lusitaniae	FSMP-Y37	FJ 627991	Traditional Egyptian Dairy Products	99	El-Sharoud*et al.* unpublished)-Egypt
YPDA5MB	*Pichia guilliermondii*	Asuan	EU182216	unknown	99	Yao et al. (unpublished)-china
	Meyerozyma guilliermondii	YZ-23	JQ 686905	Jerusalem artichoke	99	Hu *et al.* (unpublished)-china
YPDA4MB	*Meyerozyma guilliermondii*	PH-M16	GU 373754	Wastewater Treatment Systems of China	99	Yang & Wang (unpublished)-china
	Meyerozyma guilliermondii	strain LZ-1	JQ 686900	Jerusalem artichoke	99	Hu *et al.* (unpublished)-china
MM1-5	*Clavispora lusitaniae*	LZ-5	JQ 686919	Jerusalem artichoke	99	Hu *et al.* (unpublished)-china
	Clavispora lusitaniae	FSMP-Y37	FJ 627991	Traditional Egyptian Dairy Products	99	El-Sharoud*etal.*unpublished)- Egypt
M5	*Clavispora lusitaniae*	NRRL Y-11827	JQ 689030	unknown	99	Kurtzman& Robnett
	Clavispora lusitaniae	LZ-5	JQ 686919	Jerusalem artichoke	99	Hu et al. (unpublished)-china
M4	*Pichia anomala*	L80	EF116907	Wine-Related Yeast	99	Liu &Xu(unpublished)- china
	Wickerhamomyces	sp. H1Y23	JF781381	Chinese liquor fermentation-environment and materials	99	Wang T (unpublished)-china

Furthermore, PDA1 isolate was only 96% similar (more than 3% divergence) to *Pichia anomala* strain VTT C-05465 and uncultured fungi clone 28-239FM suggesting that the isolate (*PDA1*) was distantly related to both yeasts. According to Kurtzman (1998), a 0-3 nucleotide difference is considered unrelated. The phylogenetic tree (Figure 6) has indeed placed PDA1 isolate very

far from *Pichia*M4 but closer to uncultured fungi clone 28-239FM. Based on sequence alignment method as supported by the phylogenetic tree, the 9 yeast isolates were identified into 5 yeast species while isolate PDA1 did not match with any strain in the database, an indication that this isolate could be a new species.

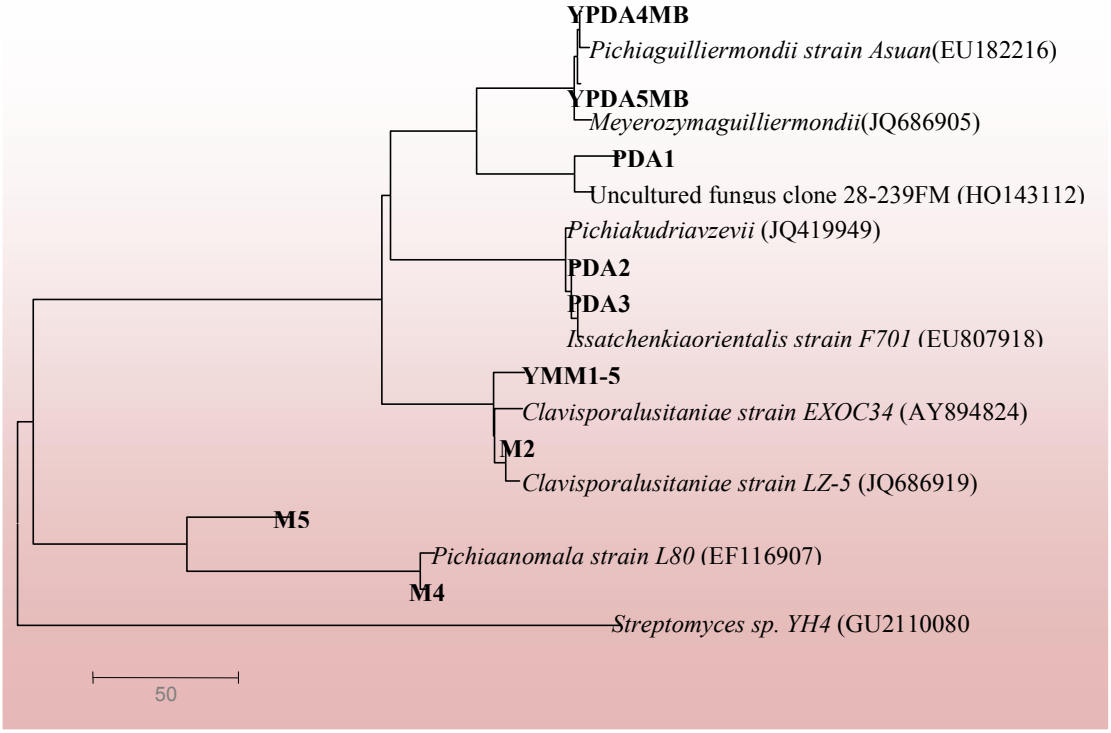

Figure 6: Phylogenetic tree showing relationship of yeast isolates using the Neighbor Joining method. (*Streptomyces* sp. YH4 (GU2110080) was used as an out-group.Evolutionary analyses were conducted in MEGA5).

Finally, BLAST results and phylogenetic tree clustered together M4 with *Pichia anomala, wine* related yeast in China. This yeast has been isolated previously by Masoud*et al.*(2004) in natural coffee fermentation environment in Tanzania and by Silva *et al.* (2008a) from green coffee storage in Brazil. *Pichia anomala* also known as *Wickerhamomyces* or *Hansenula anomala* is a non-saccharomyces wine yeast (Naumov *et al.,* 2001) and was reported to have an active beta-glucosidase enzyme that plays role in wine fermentation (Spagna*et al.,* 2002). It is likely that this attribute contributed in distinguishing isolate M4 as the best ethanol producer among all isolated yeasts reported in this study. This isolate (*Pichia anomala M4*) has not previously been indicated as ethanolic yeast in natural coffee fermentation.

Conclusion

In the coffee processing, different yeast are involved in different biochemical processes. The fermentative attributes of yeasts are well known in alcohol production although different species have instinctive capacities toward it. Isolated yeasts have had different capacities in ethanol fermentation of coffee pulp waste produced up to 3.0 % v/v and increased even further by adding sugar up to 3.56% v/v at natural conditions.This indicated that coffee processing environments have yeast flora whichare involved in ethanol fermentation and might have potential use in agro-based bioethanol fermentation.More studies are needed to establish the applicability of these yeasts for optimal bioethanol production.

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