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Selection of Mozzarella Cheese Whey Native Yeasts with Ethanol and Glucose Tolerance Ability

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***Abstract*—The research aimed to determine the native yeast on mozzarella cheese whey that has glucose and ethanol tolerance ability. The research did experimentally and the data analyzed descriptively. Native yeasts isolated from 1 ml mozzarella cheese whey with using a modification of Potato Dextrose Agar/PDA (Oxoid Ltd.) with the addition of 3% Yeasts Extract/YE (Kraft Foods) and 10 ppm amoxicillin. The yeasts identified for macroscopic and microscopic characteristics then tested with RapID Yeast Plus System. The ability in tolerate ethanol and glucose contents tested by grown the yeasts on modified Nutrient Broth/NB (Oxoid Ltd.) with 3% Yeasts Extract/YE (Kraft Foods) and 10 ppm amoxicillin then added with glucose monohydrates (10%, 20%, 30%) or ethanol (10%, 20%, 30%) and incubated for 72h at room temperature (23-28°C). Optical density (OD) read for UV absorbance at 600 nm using UV-Vis spectrophotometer every 24h until 72h. Results showed that six native yeasts isolated and identified as *C. tropicalis* three isolate, *Tri. beigelii* two isolates and *Blast. capitatus* is one isolate. The best isolates with highest OD at 30% glucose concentration (2.215) gained by *C.tropicalis* (a), while the highest OD at 30% ethanol concentration (0.508) shown by *C.tropicalis* (f).**

***Keywords*— ethanol tolerance; glucose tolerance; mozzarella cheese whey; native yeasts.**

I. INTRODUCTION

Mozzarella is one type of cheese that is now widely produced in Indonesia. High demand for this type of mozzarella cheese encouraged the Milk Treatment (MT) of South Bandung Dairy Farmers Cooperative (KPBS) to develop and produce this type of cheese. However, along with the rapid increase in production capacity, problems arise especially in managing by-products that are mozzarella cheese whey liquid.

The liquid of mozzarella cheese whey usually discharged directly into the environment and allegedly become one of the causes of pollution. Whey has a high acidity level so it will be problematic if discharged into the stream around MT KPBS. The disposal may also disrupt the balance of soil micro flora, potentially generating pathogenic microorganisms and at risk of emitting CO and CH_4 [1]. Whey discharge into the surrounding stream is potentially disruptive given the high BOD, COD and low water discharge [2] [3]. Meanwhile, on the other hand, the organic material remaining in whey has the potential to utilize.

Lactose is a specific sugar or carbohydrate contained in cheese whey, and its content can reach 4 - 5% [4]. For lactic microorganisms, lactose can act as a significant carbon source for growth. Lactic microorganisms synthesize lactose as a carbon source into glucose and galactose, then utilized through glycolysis to produce energy, organic acids and ethanol [5] [6].

Ethanol is one of the cheese whey derivatives that are potential to be developed. In addition, being used as a disinfectant, ethanol can also be used as a fuel supplement. Ethanol can act as a chemical solvent and even an important ingredient in the development of cosmetics and pharmaceutical fields.

Some types of yeast may be used to ferment ethanol from lactose contained in mozzarella cheese whey [7]. Native yeast from mozzarella cheese whey also needs to explore for its potential in fermenting lactose to ethanol. However, not all the yeast strains have resistance to stress from the ethanol they produce themselves [8]. In addition, to increase the yield of ethanol, a certain amount of sugar added thereby increasing the osmotic stress in the fermentation medium [9] [10]. This can increase the stress experienced by yeast, which ultimately inhibits ethanol fermentation.

The research aims to determine the presence of native yeast with stress tolerance ability to ethanol and high sugar concentrations is necessary. Mozzarella cheese whey as a nutrient-rich ingredient has the potential to produce native yeasts with these abilities. However, to ensure that it is necessary to identify and optimize the potential.

II. Materials And Methods

Native Yeast Isolation and Identification. Mozzarella cheese whey sample cultured on modified PDA (Potato Dextrose Agar) containing 3% yeast extract (Kraft Foods) and 100 µg per ml of oxytetracycline to inhibit bacterial growth incubate 48 hours at room temperature. The colony identified as yeast morphologically observed by microscope and sub-cultured on modified PDA then stored at 4°C [11]. Native yeast isolates with the ability to tolerate high ethanol and glucose contents identified using Remel RapID Yeast Plus System by Thermo Scientific then the results analyzed with Electronic Code Compendium (ERIC) www.remel.com/eric [8].

Stress Tolerance Test. Native yeast isolates bred in Nutrient Broth (NB) media contained 3% yeast extract (Kraft Foods) and100 µg per ml of oxytetracycline to inhibit bacterial growth, incubate 48 hours at room temperature. To test the tolerance of isolates to sugar and ethanol, yeast isolates cultured on modified NB, which had added glucose monohydrate at concentrations of 10, 20, and 30% and repeated twice. In addition, to test the resistance of isolates to ethanol content, native yeast isolates cultured on modified NB that has been added ethanol with concentrations of 10, 20, and 30%. The ability of native yeast isolates to tolerate stress on high sugar and ethanol concentrations is determined by the reading of Optical density (OD) for UV absorbance at 600 nm using UV-Vis spectrophotometer every 24h until 72h and then analyzed descriptively [12].

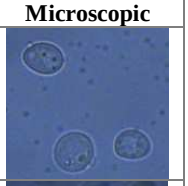
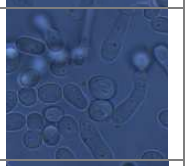
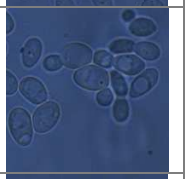
III. Results And Discussions

Native Yeasts Characterization. Six colonies were isolated from mozzarella cheese whey, identified as yeast-like microorganisms, and characterized macroscopically and microscopically (Table 1). The yeast colonies are unicellular and shaped oval, round and long, or *pseudomycelium* [13]. The colonies are grown in the media that contained amoxicillin that expected as yeasts [14]. All of colonies cell has a size of 4-8 µm, which identical with yeasts.

Mozzarella cheese whey has abundant nutrient so that microorganisms such as yeast could live. Cheese whey microbiota consists of thermophilic lactic acid bacteria with some yeast such as *Candida parapsilosis, Candida rugosa, Debaromyces hansenii, Kluyveromyces lactis, Kodamaea ohmeri, Torulaspora delbrueckii,* and *Zygosaccharomyces rouxii* [15]. The other native yeasts found in cheese whey are *Kluyveromyces marxianus, Saccharomyces cerevisiae, Clavispora lusitaniae,* and *Galactomyces geotrichum* [16]. Traditional Greek fermented whey product consists of *Zygosaccharomyces rouxii, Torulaspora delbrueckii, Debaromyces hansenii, Pichia farinosa, Candida mogii, Candida intermedia,* and *Saccharomyces cerevisiae* [17]. *Candida lambica* also found ethanol fermenting yeasts from mozzarella cheese whey [8].

Native Yeasts Identification with RapID Yeasts Plus System. RapID Yeast Plus System with the analysis of Electronic Code Compendium (ERIC) showed that the native yeast isolates identified as *C.tropicalis, Tri. beigelii* and *Blast. capitatus* (Table 2).

TABLE I
Microscopic and Macroscopic Characteristics of Native Yeasts

Isolates	Macroscopic	Size	Microscopic
a	Round -shape; concave; yellowish colony	5.23µm	
b	Round-shape; flat; white colony	4.97µm	
c	Round-shape; yellowish; small hypha	4.56µm	
d	Round-shape; small; transparent	7.94µm	
e	Round-shape; yellowish	5.44µm	
f	Round-shape; yellowish	6.72µm	

Several yeasts such as *Candida catenulata, Candida parapsilosis, Candida pararugosa, Candida tropicalis, Candida zeylanoides, Cryptococcus curvatus, Issatchenkia Orientalis, Pichia jadinii, Pichia fermentans, Rhodotorula glutinis, Rhodotorula mucilaginosa,* and *Trichosporon aquatile* were found as indigenous yeasts from milk used in the making of artisanal cheese from Quebec [18]. *Trichosporon beigelii* is often found in dairy-based products such as raw milk and Armada cheese that made from unpasteurized goat's milk [19] [20]. *T. beigelii* also found in cheese brines, which is a byproduct of cheese [21]. *Blastoschizomyces capitatus* then as *Geotrichum capitatum* is a species often described in human pathology and sourced from exposure such as contact, inhalation and foods ingestion where cheese consumption plays a major role [22].

Yeast cells require certain full scale and microelements for development, digestion, and cell steadiness. Magnesium and calcium are macroelements. Magnesium constitutes 0.3% of the cell dry weight and goes about as a compound activator (particularly for all synthetases, phosphatases, and kinases) and a pressure silencer, and it controls cell division, development, and size [23] [24] [25]. It checks the harmful impacts of Cu, Co, Cd, and Al. Magnesium has been accounted for to direct metabolic proteins of the fermentative pathway (through pyruvate decarboxylase) or

the respiratory pathway (using pyruvate dehydrogenase) and the exchanging amongst respiratory and fermentative procedures [26] [27]. In the meantime, calcium is engaged in directing amylase movement and phosphate precipitation and furthermore assumes a defensive part for cell films [28].

Fermentative yeast has an appeal for Mg due to glycolytic chemical action, and free intracellular accessible Mg may not be adequate to satisfy the prerequisite. Additionally, the connection of Mg and Ca is adversarial. Calcium influences the take-up and bioavailability of magnesium. Calcium restrained many transphosphorylases of glycolysis that fortified by Mg [25]. Industrial fermentations might control by supplementing yeast media with magnesium salts, particularly MgSO4. In this way, the change of the Mg/Ca proportion in yeast maturation media will prompt enhance liquor generation because mechanical yeast media do not meet the cell interest for Mg and Ca. Previous research has shown the highest ethanol yield (12.53% v/v) with a 2:1 Mg/Ca proportion alongside a mix of Zn meanwhile magnesium also could enhance biomass and ethanol generation with xylose [29] [30].

TABLE II
THE RESULTS OF RAPID YEASTS PLUS SYSTEM WITH ERIC ANALYSIS

Isolate	**a**	**b**	**c**	**d**	**e**	**f**
Glucose	+	-	-	-	-	+
Maltose	+	-	-	-	-	+
Sucrose	+	+	-	-	+	+
Trehalose	+	+	-	-	-	+
Raffinose	-	-	-	-	-	-
Lipid	-	-	-	-	-	-
NAGA	+	-	-	-	-	-
αGlucoside	+	+	+	+	+	+
bGlucoside	-	-	+	+	-	-
ONPG	-	-	-	-	-	-
αGalactoside	-	-	+	+	-	-
bFucoside	-	-	+	+	-	-
PHS	-	-	-	-	-	-
PCHO	-	-	-	-	-	-
Urea	-	-	+	+	-	-
Prolyne	-	-	+	+	-	-
Histidine	+	+	+	+	+	+
Leucyl-Glycine	+	+	+	+	+	+
Yeast Name	***C.tropicalis***	***C.tropicalis***	***Tri.beigelii***	***Tri.beigelii***	***Blast.capitatus***	***C.tropicalis***

Stress tolerance towards Ethanol and Glucose. All of the isolates have shown the ability to tolerate stress caused by high concentration of ethanol until 48-h (Fig. 1).

Ethanol is dangerous to yeasts since it can hinder yeast development as the fixation increments in the substrate [31]. Ethanol can devastate mitochondrial DNA inside the yeast cells at that point causing unsettling influence and may diminish development rate, fermentation rate and cell feasibility of yeast that came about by inactivation of hexokinase and dehydrogenase enzyme [32]. Yeasts capacity to make due in a substrate with high ethanol contents has found to be 14% - 20% [33]. The capacity identified with the fatty acid synthesis controlled by the yeast cell walls [34].

Ethanol resilience in yeast is an intricate phenotype, as it affected by accessible supplements and development substrates, and additionally by ecological factors, for example, temperature and osmotic weight [35] [36]. One instrument that has developed in intervening both ethanol and temperature resilience is the plasma layer structure and film smoothness. Film ease affected by phospholipid and sterol structure, as well as by ethanol and temperature [37] [38]. Besides, the lipid organization has found to assume an essential part in both ethanol and thermal resilience. Both ethanol and temperature resistance have been fundamental to connected examinations of ethanol synthesis by yeast [39] [40].

Gene has additionally been distinguished from exploratory advancement with determination for high ethanol [41]. Ethanol tolerances also are known to be temperature subordinate [35]. Earlier researches have either centered on hereditary variety in either ethanol or temperature push however not both [42] [43] [44]. The two crosses contrasted in the other parent (YJF153 or SD1) and the impacts of the sensitive SEC24 and PSD1 alleles from HN6. In the HN6 x SD1 cross, the HN6 SEC24 allele, presented affectability to warm and more prominent to warmth and ethanol consolidated. In the HN6 x YJF1533 (Oak) cross, the HN6 SEC24 allele just made affectability warmth and ethanol consolidated through the HN6 allele of PSD1 made affectability warm alone. Curiously, the affectability of HN6 was not as extreme in the haploid contrasted with the diploid form, which has caused by a measurement impact [41].

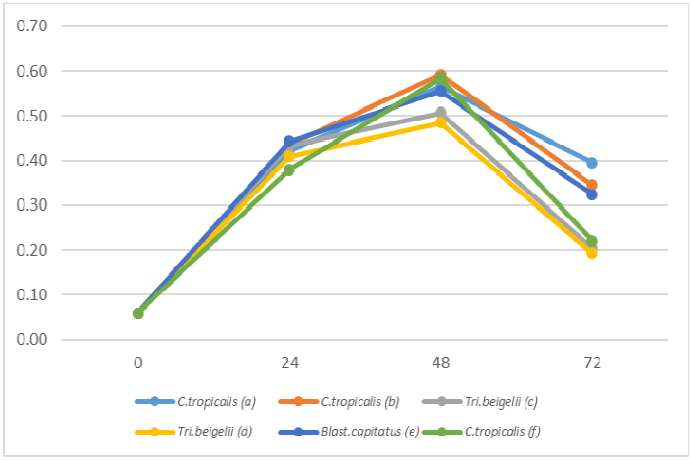
HN6 x YJF153 recombinant strains are bearing the PSD1 delicate allele, demonstrating that alleles from the YJF153 are important for the articulation of PSD1 warm resilience.

The HN6 alleles affected to a solitary amino corrosive substitution in PSD1 and both of two substitutions in SEC24. The phenotypic impacts of corrosive amino substitutions in SEC24 point to its significance in the joined resistance to ethanol and high temperature. In any case, because SEC24 is a fundamental quality, it is likewise conceivable that the corrosive amino substitutions result in temperature touchy alleles of SEC24, and that SEC24 is not intrinsically associated with ethanol and warmth resilience. SEC24 alleles do not affect affectability to warm alone in one of the two crosses (HN6 x YJF153) does not bolster this later understanding. Another potential component of SEC24 intercede affectability to ethanol and warmth is its part in ER to Golgi transport. ER to Golgi transport is an essential segment of protein quality control; misfolded proteins in the ER transported once again into the cytoplasm keeping in mind the end goal corrupted by the ubiquitin-proteasome framework [46].

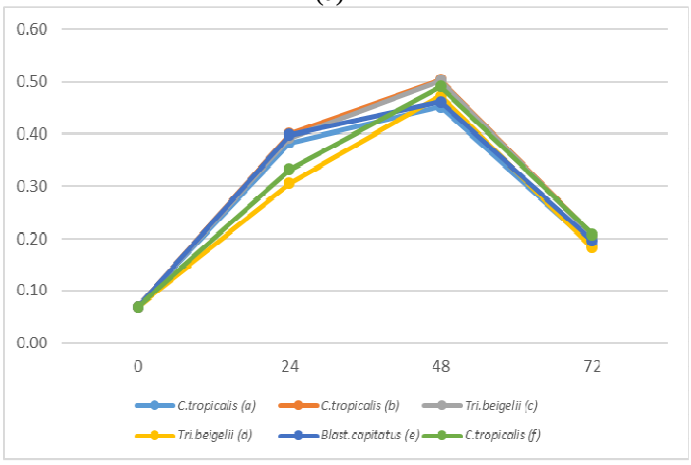
While it is not clear why the SEC24 allele from HN6 is especially touchy to warm within sight of ethanol, this phenotype might be interceded by surrenders in the vehicle of proteins vital to warmth and ethanol resilience or to deserts in the Golgi or ER layers themselves. The instrument by which PSD1 influences warm affectability likely identified with its effect on mitochondrial layers, however, relies upon other hereditary variables originating from the YJF153 foundation. PSD1 changes over *phosphatidylserine* to p*hosphatidylethanolamine* (PE), a mitochondrial phospholipid that assumes an essential part in mitochondrial combination and in the support of mitochondrial morphology [47]. Mitochondrial work is known to be vital for inherent warmth obstruction and cancellation of two qualities, CHO1 and OPI3, required for change of PE to *phosphatidylcholine* brings about warmth stun affectability [48]. Besides, it has recommended that warmth instigated changes in layer ease impact the impression of high temperature and the outflow of warmth stun proteins [49]. While PSD1 has not already distinguished as a quality giving protection from high temperatures, this might be a result of its reliance on obscure hereditary variables isolating in the HN6 x YJF153 recombinants. Overexpression of PSD1 and SEC24 did not improve warmth or warmth and ethanol resistance, and on a few occasions was dangerous. The lethality in delicate strains and inability to improve development in safe strains could cause by the way that SEC24 is one of five fundamental proteins that shape the COPII vesicle coat. It alongside SEC23 shapes the inward layer of the vesicle as a heterodimer and ties the load that will transport from the harsh Endoplasmic Reticulum to the Golgi mechanical assembly [50].

High duplicate articulation of SEC24 may prompt an excess of the protein, which thus may block heterodimer arrangement. It has been demonstrated that overexpression of both SEC24 and SEC23 prompts diminished development and diminished development rate in yeast. [51] [52].

Non-Saccharomyces yeasts, for example, *Hanseniaspora* and *Candida* mostly not tolerant to 4-6% ethanol, anyway late research demonstrate the ethanol-resistance capacity like *S.cereviseae* [53] - [55]. *H.guilliermondii* and *C.krusei* can deliver unsaturated fats in the plasma layer as the systems to adjust the nearness of ethanol stretch [56]. The expansion in the extent of ergosterol or oleic corrosive in *H.guilliermondii* cells gives an incredible adjustment to high ethanol focus [57]. *C.krusei* as *I.orientalis* could increment caprylic corrosive, stearic corrosive associative with the diminishing of oleic corrosive and palmitoleic acids to endure high ethanol focus [56].



(a)



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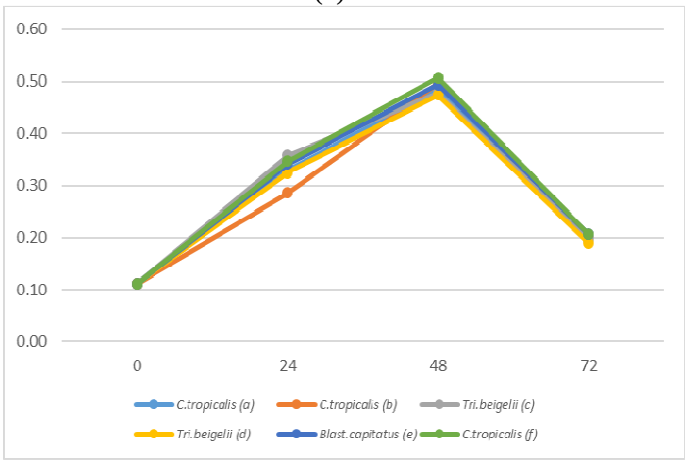
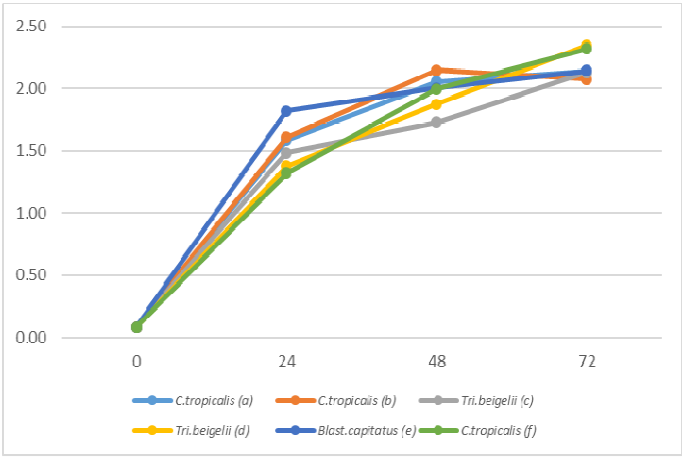
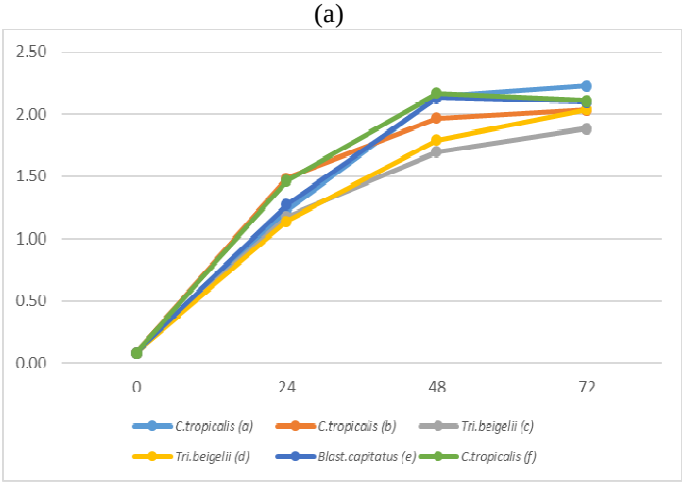


(c)

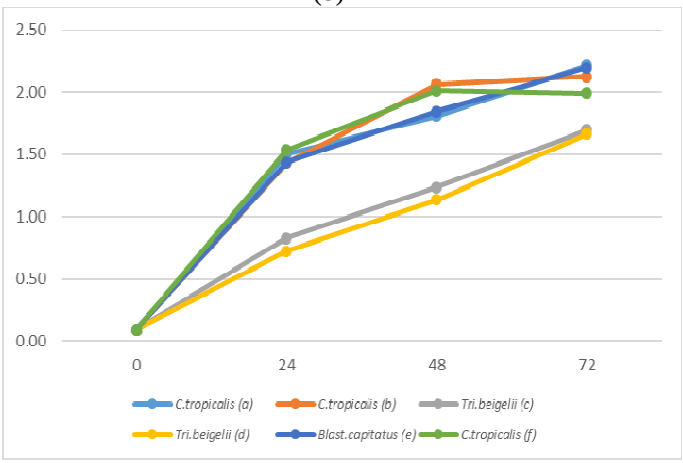
Fig. 1. Native yeasts tolerance towards (a) 10% Ethanol; (b) 20% Ethanol; (c) 30% Ethanol



(a)



(b)



(c)

Fig. 2. Native yeasts tolerance towards (a) 10% Glucose; (b) 20% Glucose; (c) 30% Glucose

The ethanol fixation expansion influences the cell layer respectability and film penetrability. Various ionic species will harm and plasma layer smoothness likewise will diminish, which annoys protein adaptation of glycolytic chemicals (pyruvate kinase and hexokinase) at that point influences the take-up of glucose, maltose, ammonium, amino acids, and furthermore causes cell spillage of nucleotides, amino acids, and potassium particles that appeared by the aggravation of yeasts development [58]. Yeasts cell has created in adjusting the expansion of ethanol focus through the difference in film organization against layer fluidization and plasma film adjustment [56].

It appeared stress resistance towards high centralization of glucose segregates until 72-h (Fig. 2). High glucose focus is one factor that can repress the development of yeast. The sugar centralization of 20-30% could diminish the yeast development rate as showed by the lessening in silt shaped from all detaches [59] [60]. The high centralization of sugar prompts high osmotic weight, which causes low levels of yeast development. [61]. In any case, a few yeasts having the capacity to blend and use glycerol may hold on in substrates that have high osmotic weight because of high sugar focuses [62].

Osmotolerant yeasts can expend glucose and combination glycerol with low corrosive generation toward the start of aging [8]. Proficient in glycerol transport into yeasts cell is a fundamental instrument in fighting osmotic pressure came about because of high glucose, focuses [63]. Non-*Saccharomyces* yeasts, for example, *Candida* and *Hanseniaspora* could survive high glucose push in light of the capacity to absorb succinic and acidic corrosive that came about because of osmotic pressure condition [64]. *Candida spp.* were indigenous yeasts that generally found on commonly or intentionally ethanolic aging and furthermore can endure high convergences of ethanol [65] [66].

IV. Conclusions

Results showed there are six native yeasts isolated and identified as *C. tropicalis* three isolates, *Tri. beigelii* two isolates and *Blast. capitatus* one isolates. The best isolates with highest OD at 30% glucose concentration (2.215) gained by *C.tropicalis* (a), while the highest OD at 30% ethanol concentration (0.508) shown by *C.tropicalis* (f).

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