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Isolation and Identification of Osmophilic Yeasts Isolated from Molasses Sugarcane as Bioethanol Starter

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***Abstract*— Osmophilic yeasts was second generation to increase of the bioethanol production. The yeasts were isolated from the high sugar ontain material like honey, juice or by-product materials such as molasses. Bioethanol was usually produced from sugarcane molasses at brix degree of 14%. The aim of the research Reviews These were isolation and identification of phenotypic identification of indigenous-molasses yeast. A phenotypic characteristics were based on typical colony, morphological and fermentation profile. The yeasts were isolated from original molasses (> 80% brix) on malt extract agar (MEA). There were two isolates of osmophilic yeasts. Isolate A regular was clear white colony, sharpe cell with multilateral budding, while isolate B was murky white colony regular, cylindrical cell with multilateral budding. Isolate A and B optimum grown at 30°C and produce high ethanol at 34° Brix but the pH of the growth of yeast were difference. Isolate A growth optimum at pH 5, while isolate B optimum growth at pH 4. The fermentation profile using the API 20C Aux kit Showed that the yeasts can Ferment glucose, glycerol, calcium-2-keto-gluconate, arabinose, adonitol, galactose, sorbitol, methyl-αD-glucopiranoside, N-acetyl-glucosamine, Cellobiose, maltose, sacharose, trehalose, and raffinose. Isolate A Ferment can inositol and melezitose, while isolate B can Ferment xylose and xylitol. Isolate A was identified as *Candida famata* (63.2%) and B isolates was identified as *Candida guilliermondii* (84.3%).**

Keywords—bioethanol fermentation, osmophilic yeast, sugarcane

I. INTRODUCTION

M*olasses* used as a medium for the production of commercial alcohol on alcoholic fermentation industry, because it is easily obtained widely, cheaply and as the quality of raw materials [1]. Industry uses a substrate with a sugar concentration is 16-18%, if the sugar concentration is higher than 18% will cause osmotic pressure which reduces the efficiency of the fermentation process [2].

In the bioethanol industry, the sugar concentration on the substrate used is 16-18%, and if it is higher than 18% will cause osmotic pressure which reduces the efficiency of the fermentation process, so as to isolate the species or strain of yeast osmofilik origin of molasses that are resistant to high osmotic pressure by characterizing the morphology, physiology and molecular identification. Several studies have isolation of yeast osmofilik of Indonesian food such as honey, milk and fruit jams, obtained yeast isspecies *Candidametapsilosis,* C. *etchelsii,* C. *parapsilosis,* C. *orthopsilosis* and *Sterigmatomyces halophilus* [3].

This study aimed to isolate the origin osmofilik molasses yeast, characterize morphology and physiology as well as identify the molecular origin of yeast osmofilik molasses. Yeast osmofilik molasses origin was expected to be applied to the production of bioethanol with high brix sugar cane molasses, or more than 14 °. It is hoped that the yeast will have a good adaptability to the production of molasses alcohol with the substrate.

II. METHODS

A. Materials

Molasses was take from PG. Jatiroto sugarcane milling at season 2015. The molasses was prepared by taking a sufficiently concentrated molasses and molasses brix measured 14°, 24° and 34° using a refractometer. Molasses brix 34° was made of 200 ml of concentrated molasses and diluted with 350 mL of distilled water. Molasses brix 24° was made of 200 ml of concentrated and diluted with 555 mL of distilled water. Molasses brix 14° was made of 200 ml of concentrated and diluted with 935 mL of distilled water.

B. Isolation Yeast

Isolation of molasses indigenous yeasts was carried out by taking 1 ml of concentrated molasses and spread on a solid MEA media with dispersive method at 2 petridish. Every isolates were grown from each petridish, purified on MEA solid media with a 3x quadrant scratch method. Isolates were purified, scratched back on a slanted MEA media as saving culture and work culture.

C. Morphological characterization

Morphological characterization was done by macroscopic and microscopic observation. Macroscopic observation was conducted by observing the yeast colonies that grow directly on the surface of the MEA media, including colony shape and color of the colony. Microscopic observations done by creating a wet mount. Wet mount was made to fix the yeast cells on a *glass* object,given thedye, *crystal violet*and do repainting with a mordant. The final stage, was observed under a microscope magnification of 1000X with the addition of immersion oil and covered with a *cover*glass. Microscopic observations made include cell shape, presence *cell budding* and type of *budding* cell.

D. Physiology Characterization

Physiological characterization of yeast was conducted on the test temperature and pH growth, endurance test molasses brix grow at 14°, 24° and 34°, and analyse of alcohol production. Profile of fermentation was analyzed by using API 20C-Aux Kit.

1) Test of yeast growth temperatures

This test was performed by taking 0.03 ml of 3 ml of yeast culture MEB age of 48 hours, then inoculated in 1 ml of sterile MEB media. Each yeast culture was incubated at different temperatures: 10°C, 20°C, 30°C, 40°C and 50°C for 48 hours. Observations yeast growth at any temperature, was done by measuring the absorbance values of each yeast culture using a spectrophotometer at OD 600 nm on incubation time of 12 hours, 24 hours, 36 hours and 48 hours.

2) Test of yeast growth pH

This test was performed by taking 0.03 ml of 3 ml of yeast culture MEB age of 48 hours, then inoculated in 1 ml of sterile MEB medium with a pH of 3, 4, 5, 6. Each yeast culture was incubated at 30°C for 48 hours. Observation of yeast growth on any media pH MEB, carried out by measuring the absorbance values of each yeast culture using a spectrophotometer at OD 600 nm on incubation time of 12 hours, 24 hours, 36 hours and 48 hours.

3) Test of yeast growth on molasses at brix 14°, 24° and 34 °

Test was done by taking a 0.03 mL of work culture of yeast in 3 ml MEB age of 48 hours, then inoculated in 3 ml of sterile molasses brix 14 °, 24 ° and 34, and incubated at 30°C for 24 hours and 48 hours. Growing endurance test was conducted using the scatter. On the control, yeast culture in 3 ml MEB life of 48 hours, inoculated in 3 ml physiological saline. Furthermore, the yeast culture was taken as 1 ml and diluted in 9 ml physiological saline solution at a dilution series of 10^{-1} to 10^{-7}. Yeast culture (age of 24 hours and 48 hours) was taken as 1 ml and inoculated at 9 ml physiological saline solution and diluted with a series of 10^{-1} to 10^{-7}. Dilution series of 10^{-4},10^{-5},10^{-6} and 10^{-7} in the control and treatment groups was taken each of 1 ml, plated on solid MEA medium and incubated at 30°C for 48 hours. Yeast colonies growing on each plate, count the number of colonies compared to the control (Log CFU / ml).

4) Fermentation Test Pattern

This test was performed using the API 20C-Aux kit, consisting of 20 wells were wells first as a negative control (O), pitting both as a positive control containing glucose (GLU), and 18 sinks more were glycerol (Gly), calcium 2-keto-gluconate (2KG), arabinose (ARA), xylose (XYL), adonitol (ADO), xylitol (XLT), galactose (GAL), inositol (INO), sorbitol (SOR), methyl-αD-glucopyranoside (MDG), N- acetyl-Glucosamine (NAG), Cellobiose (CEL), lactose (LAC), maltose (MAL), saccharose (SAC), trehalose (TRE), melezitose (MLZ) and raffinose (RAF). Fermentation profile was performed by standard procedures (bioMerieux, 2010). Observations pattern of fermentation after 48 hours and 72 hours was based on whether or not the substrate turbid sugar on pitting. If the media becomes cloudy, it mean a positive reaction and if the media was clear (not cloudy), it mean negative reaction. The results were written on a *sheet* result, accumulated and acquired on 7 digit biocode. The code was compared with the identification book or dikonfimasi on apiweb^{TM} (https: //apiweb.biomerieux.com//) to identify the type of yeast.

III. RESULTS AND DISCUSSION

A. Morphological Characteristics

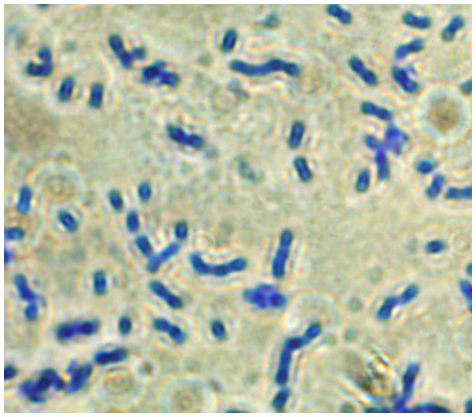
Isolation of osmophilic-molasses indigenous yeasts obtained two isolates that isolates 1 on the first petridish was coded A and isolates 2 on the second petridish was coded B. Purification of isolates was conducted by using quadrant scratch with three times. Single colony was purified again using the scratch method as savings culture and work culture.

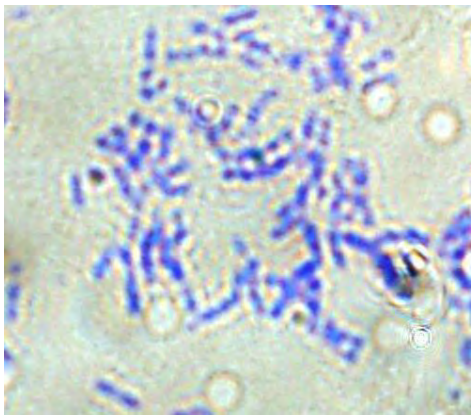
Macroscopic characteristics of yeast was based on the appearance of colonies grown on a solid medium, including textures, shapes, colors, margins, elevation and the surface of colony. Microscopic charactertics included cell shape, cell size, formation of *budding,* sprouting type *(budding cell),* and the presence or absence pseudohyphae/hyphae [4,5]. Microscopic observations used observation under the microscope Leica DM 2500, 1000x magnification by addition of emersi oil. Macroscopic and microscopic characters of yeast isolates A and B were shown in Table 1.

Microscopic characteristics of isolate A were round cell shape, cell budding with multilateral type. While the microscopic characteristics of isolate B were cylindrical cells, cell budding with multilateral type. Microscopic characteristics of isolate A and B were shown in Figure 1.

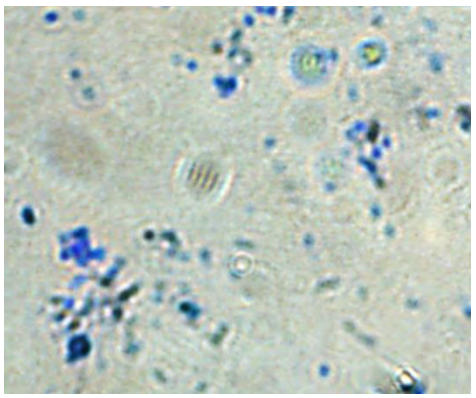
Table 1 Morphological characteristics of A and B isolates

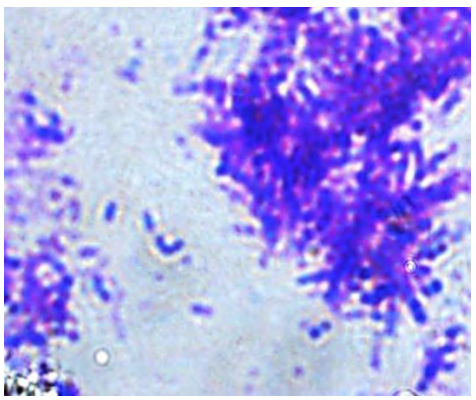
Isolates Yeast	Macroscopic characteristics		Microscopic characteristics		
	Shape of colonies	Colonies color	Cells form	Budding	budding type
A	Bulat	White	Bulat	There	Multilateral
B	Round	white	Cylinders	there	multilateral





Isolate after 20 h incubation





Isolate after 40 h incubation

Figure 1. Microscopic characteristics of isolate A and isolate B after 20 h and 40 h incubation at 400x magnification

B. Physiology Characterization

The isolate A can good growth at 20 - 30°C, while the isolate B can good growth at 30-40°C, but the both of isolate can optimum growth at 30°C. Fardiaz [6] reported that the optimum growth at 25-30°C. Figure 2 and Figure 3 showed the growth of yeasts isolate A and B at some growth temperature (20ºC, 30ºC, 40ºC, 50ºC).

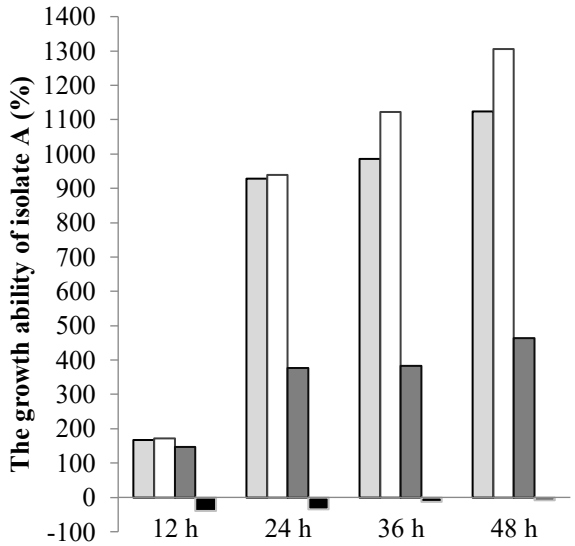


Figure 2. The growth ability of isolate A on MEB medium at at temperature: 20°C (▭), 30°C(▭),40°C(▭) and 50° C(▭).

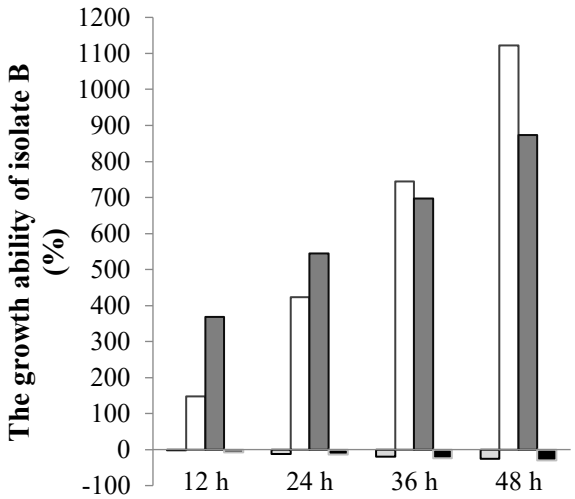


Figure 3. The growth ability of of yeast isolate B on MEB medium at temperature: 20°C (▭), 30°C (▭),40°C (▭) and 50 ° C (▭).

The yeasts isolates A and B able to growth at pH 3, 4, 5, 6 (Figure 4 and Figure 5). Both isolates had the optimum growth pH i.e pH 5. Prescott and Dunn [7] reported that the yeast growth pH was between 3.0 to 6.0.

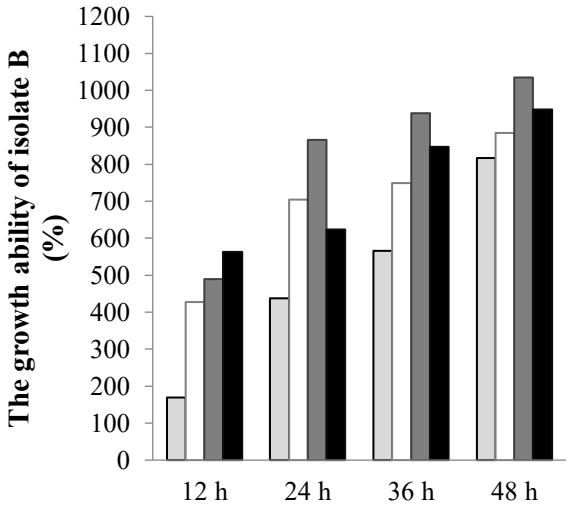


Figure 4. The growth ability of of yeast isolate A on MEB medium at pH: 3(□),4 (□), 5 (▩), and 6 (■).

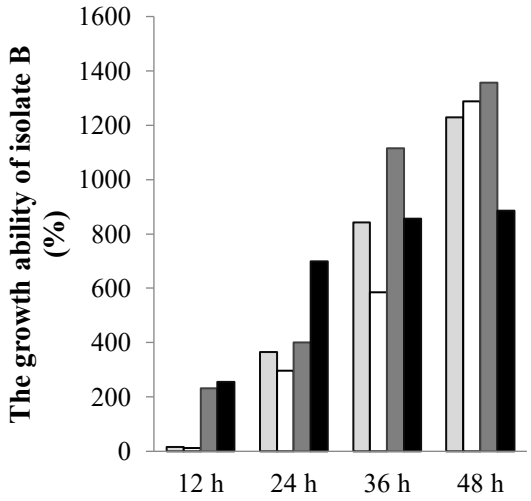


Figure 5. The growth ability of of yeast isolate B on MEB medium at pH: 3°C (□),4°C (□), 40°C (▩) and 50 ° C(■).

The isolate A and isolate B were able to grow on molasses brix 14 °, 24 ° and 34 °. The highest yeast population were at 34 ° brix molasses i.e 6.91 Logs CFU/ml for isolate A at 48 h incubation and 6.27 Logs CFU/ml for isolate B at 24 h incubation. The yeast population at 24 ° molasses brix were 6.24 log CFU/ml for isolate A at 48 h incubation and 6.23 log CFU/ml for isolate B at 24 h incubation. The yeast population at 14 ° molasses brix were 6.86 log CFU/ml for isolate A at 48 h incubation and 6.20 log CFU/ml for isolate B at 24 h incubation. Isolates A and B the highest growth at 34° brix molasses, because it has the highest sugar concentration than molasses brix 14° and 24°. It indicated that the isolate A and B were osmophilic yeast.

Isolates A increased the highest growth at 48-hour incubation. That was in accordance with the fermentation time needed for fermentation is 2-3 days. Isolates B increased the highest growth at 24-hour incubation. After 20 hours, it was optimum use of sugar by yeast to produce the primary metabolite (ethanol). The ability to grow isolates A and B on molasses brix 14°, 24° and 34° can be reached 6,17 Log CFU/ml (0 hours), resulting in an increase to the highest growth (3.15%) for isolate A and (1.60%) for isolate B (Figure 6 and Figure 7).

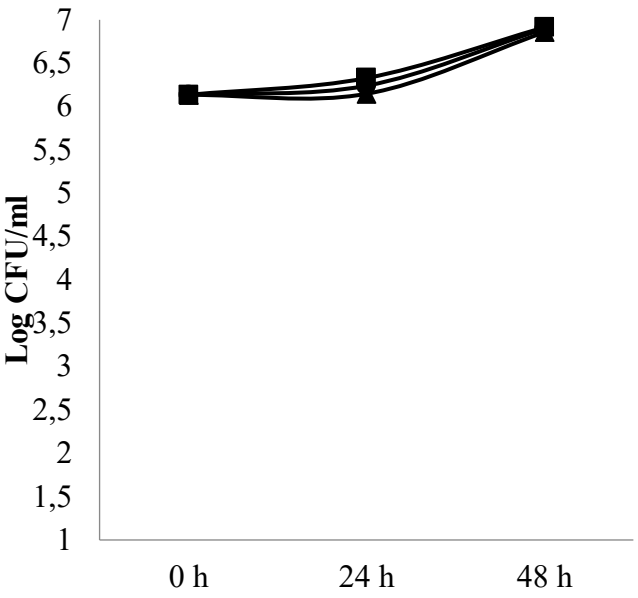


Figure 6. The growth ability of isolate A media molasses brix: 14 ° (▲), 24 ° (●), 34 ° (■) for 24 h and 48 h incubation

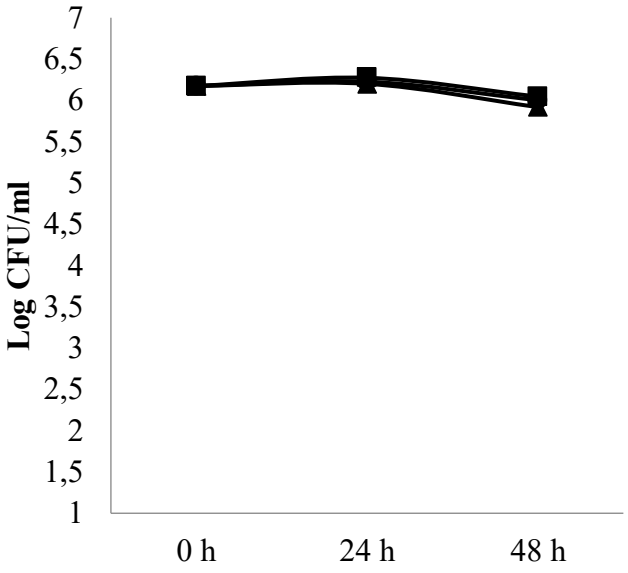


Figure 7. The growth ability of isolate B media molasses brix: 14 ° (▲), 24 ° (●), 34 ° (■) for 24 h and 48 h incubation

Evaluation of yeast profile fermentation were determined by incubate the isolates at 20 C Aux API kit for 72 hours. A and B isolates were grown on 18 kinds of media on the strip kit API 20C Aux. Results obtained in the form of negative or positive reactions, indicated by cloudy or absence of media after 72 hours of incubation. A positive reaction, if the isolates were inoculated make media becomes cloudy, it means that isolates A or B can ferment the carbon source in the kit

substrate and the negative reactions if the kit substrate was clear which means the yeast isolate was not able to ferment the kit substrate. The result showed that isolates A can ferment some sugars such as GLU, Gly, 2KG, ARA, ADO, GAL, INO, SOR, MDG, NAG, CEL, MAL, SAC, TRE, MLZ, RAF on Aux kit API 20C, so that the reaction is positive (+). Isolates A can not ferment kind sugar kind XYL, XLT and LAC on the kit API 20C Aux, and have no *hyphae* or *pseudohyphae,*so that the reaction is negative (-). The results were as shown in Table 2.

Results of a positive or negative reaction of the isolates A, was determined to be 7 biocode i.e 6 3 5 7 3 7 3 after incubation for 72 hours. The code was a code to identify isolate A. Code 6 3 5 7 3 7 3 confirmed on programe web to determine the species of osmophilic yeast. The identification result sreported that isolate A was *Candida famata* (with identify confidence 63.2%). According Dmytruk and Sibirny [8], carbon assimilation test for C. *famata* that was positive assimilate glucose, galactose, maltose, sucrose, trehalose, D-xylose, melezitose, glycerol, raffinose, Cellobiose, L-arabinose; possibility assimilate lactose, starch dissolved, L-rhamnose, D-arabinose; cannot assimilate potassium nitrate and inositol. According Santra [9], *C. famata* isolated from palm wine beverage, characterization morphological and biochemical tests. The results obtained by the *Candida famata* can assimilate D-glucose, D-galactose, D-xylose, sucrose, maltose, α, αTrehalose, Cellobiose, lactose, raffinose, and Glycerol; while unable to assimilate Myo-inositol, 2-Keto-D-glucose, starch and L-ramnose. Isolates A has some similarities with the characteristic C. *famata* above, which was able to assimilate D-glucose, D-galactose, maltose, sucrose, trehalose, melezitose, glycerol, raffinose, Cellobiose, L-arabinose, and did not have the *pseudohyphae* or *pseudomycellium.* The isolate A have the characteristics of some differences with C. *famata* above, isolates still incapable of assimilating inostitol and unable to assimilate D-xylose and lactose; while C. *famata*a can assimilate D-xylose and lactose; and can not assimilate inositol.

The isolate A was *doubtful profile* so isolate A still needed to be further identified by molecular identification. Conventional identification based on morphological, physiological and biochemical, can lead to misidentification especially in closely related species [10]. The isolate A were identified only 63.2%, which still requires the identification of more accurate, and require the identification of yeast easily, quickly and accurately the method of molecular identification.

Table 3 showed that isolate B can ferment several types of sugars, such as GLU, Gly, 2KG, ARA, XYL, ADO, XLT, GAL, SOR, MDG, NAG, CEL, MAL, SAC, TRE, RAF the kit API 20C Aux, so that the reaction is positive (+). Isolates B can not ferment sugars, such as INO and LAC on the kit API 20C Aux and have no *hyphae* or *pseudohyphae*so that the reaction was negative (-).

Table 2. The fermentation profile of isolates A in Auxkit API 20C

	sugar type kit API 20C Aux																				
CODE	O	GL	Gly	2K	AR	XY	AD	XL	GA	INO	SO	MD	NA	CE	LA	MA	SA	TR	ML	RA	H/
A	-	+	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-

Table 3. The fermentation profile of isolates B in Auxkit API 20C

	kit API 20C sugar type Aux																				
CODE	O	GLU	Gly	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	H / PH
B	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-

Results of positive or negative reaction from isolates B, was determined to be 7 biocode ie 6 7 7 6 3 7 3 after incubation for 72 hours. The results showed that isolate B was *Candida guilliermondii* (84.3%) with identifying information *excelent identificaton to the* genus. According to Ogba *et al* [11] species characterization *Candida* was performed using kit identification *Candida microexpres* a test for the assimilation and fermentation of sugar. One of the species *Candida* that characterized that C. *guilliermondii* can ferment melibose, sucrose, galactose, cellibiose, xylose, raffinosa and dulcitol, but cannot utilize sugars such as lactose, maltose and inositol. Isolate B had some similarities with the characteristics of C. *guilliermondii* above, which was able to assimilate sucrose, galactose, cellibiose, xylose, raffinosa and cannot assimilate lactose, inositol. Isolate B had differences with *C. guilliermondii* that assimilate maltose whereas the isolate B cannot assimilate maltose. Conventional identification based on morphological, physiological and biochemical, can lead to misidentification especially in closely related species [11, 12].

IV. CONCLUSION

There were obtained two osmophilic yeast isolated from sugarcane molasses. Morphological characteristics of isolate A and B were round colonies, colony color white and rounded cell shape and type of multilateral budding; while isolate B had cylindrical cell shape and type of multilateral budding. Optimal physiological characteristics isolate A grew at 30 °C and pH 5; had the ability to grow on a substrate molasses brix 14 °, 24 ° and 34 °; and able to ferment glucose, glycerol, calcium-2-keto-gluconate, arabinose, adonitol, galactose, inositol, sorbitol, methyl-αD-glukopiranosida, N-acetyl-glucosamine, cellobiosa, maltose, sucrose, trehalose, melezitose, raffinosa. Isolate B grew optimally at 30 ° C and pH 5; had the ability to grow on a substrate molasses brix 14 °, 24 ° and 34 °; and able to ferment glucose, glycerol, calcium-2-keto-gluconate, arabinose, xylose, adanitol, xylitol, galactose,sorbitol,methyl-αD-glukopiranosida,N-acetil-glucosamine, cellobio sa, maltose, sucrose, trehalose, raffinosa. Isolate A was identified as *Candida famata* (63.2%) and isolate B was identified as *Candida guilliermondii* (84.3%).

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