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Isolation and Characterization of Ethanol and Thermotolerant Yeast Isolates from Different Sources in Atbara Town – River Nile State - Sudan

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ABSTRACT

Thirty yeasts isolates were isolated from five fruits namely grapes (*Vitis vinifera*), apple (*Malus domestica*), date palm (*Phoenix dactylifera*), banana (*Musa paradisiaca*) and fermented sorghum dough (Ajeen) collected from Atbara market. The viable cell count of yeasts isolates was enumerated. All the yeast isolates were first screened for carbohydrate fermentation using Durham tube fermentation method in yeast extract peptone dextrose broth. Five isolates (SUDA, SUDV, SUDMU, SUDP and SUDDD) which were relatively high fermentative were selected for further study. All the selected isolates were identified morphologically, using macroscopic and microscopic features. The yeast isolates were also screened for ethanoland thermo - tolerance. Further, the optimum pH was determined. The results of this investigation revealed that the yeast colony forming unit was ranged from 99 x104 cfu/ml to 118 x104 cfu/ml. For the test of temperature, growth was detected up to 40°C with optimum temperature at 37°C for all isolates, for ethanol concentration 15% was optimum for all isolates except SUDMU and SUDP was at 20% and 25%, respectively. The optimum pH was ranged from 5-6. The aim of this study is to isolate and characterize ethanol, and thermo - tolerant yeast for industrial purposes.

Keywords: Fruits, Sorghum dough, yeast isolates, thermo and ethanol tolerant

عزل وتوصيف الإيثانول وعزلات الخميرة المقاومة للحرارة من مصادر مختلفة في مدينة عطبرة ـ ولاية نهر النيل ـ السودان

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مُسْتَخْلَص

تم عزل ثلاثين من عزلات الخميرة من خمس فواكه وهي العنب*(Vitis vinifera)* والتفاح *(Malus domestica)* ونخيل التمر*(Phoenix dactylifera)* والموز*(Musa paradisiaca)* وعجينة الذرة الرفيعة المخمرة (Ajeen)التي جمعت من سوق عطبرة. تم إحصاء عدد الخلايا الحية لخمائر العزلات. تم فحص جميع عزلات الخميرة لأول مرة لتخمير الكربوهيدرات باستخدام طريقة التخمير الأنبوبية Durham في مستخلص الخميرة من مرق البيتونديكستروز. تم اختيار خمس عزلات (SUDA و SUDV و SUDMU و SUDP و SUDDD) والتي كانت عالية التخمير نسبيًا لمزيد من الدراسة. تم التعرف على جميع العزلات المختارة شكليا باستخدام الخصائص العيانية والميكروسكوبية. كما تم فحص عزلات الخميرة لمعرفة الايثانول والمقاومة للحرارة. علاوة على ذلك، تم تحديد الرقم الهيدروجيني الأمثل. أظهرت نتائج هذا البحث أن وحدة تكوين مستعمرة الخميرة تراوحت من 99x 104 cfu / ml إلى 118 **x** 104 cfu/ ml لاختبار درجة الحرارة، تم الكشف عن نمو يصل إلى 40 درجة مئوية مع درجة الحرارة المثلى عند 37 درجة مئوية لجميع العزلات، بالنسبة للإيثانول فإن تركيز 15٪ كان الأمثل لجميع العزلات باستثناء SUDMU و SUDP كان عند 20٪ و 25٪ على التوالي. تراوح الرقم الهيدروجيني الأمثل بين 5-6. تهدف هذه الدراسة إلى عزل وتوصيف الإيثانول والخميرة المقاومة للحرارة للأغراض الصناعية.

الكلمات المفتاحية: *فواكه، عجينة الذرة ، عزلات الخميرة ، مقاومة للحرارة و الإيثانول*

Introduction

Production of ethanol by fermentations is among the most ancient processes known. Yeasts are used in the fermentative production of ethanol, alcoholic beverages, baking products, protein and vitamin supplements in human and animal diets as well as in the production of single cell proteins (Moneke *et al*., 2008). Yeasts are the safest and most effective microorganisms for fermenting sugars to ethanol and traditionally have been used in industry to ferment glucose based agricultural products to ethanol (Ho *et al*., 1998). Yeast is ubiquitous in the environment, but is most frequently isolated from sugar rich samples (Haggran, and Abo-Sereih, 2014). Moreover, thermophilic and thermotolerant microorganisms are important in the industry. The use of such microbes in the industry will lead to reduction in the cooling cost. These microbes could be used directly in the fermentation industry (Mustafa *et al*., 2017). Alcoholic fermentation processes leads to a decrease in oxygen solubility as the process temperature increases, thus microbial involvement requires candidates that function under anaerobic conditions and accumulation of ethanol inhibit the yeast growth and consequently stop the fermentation process (Cakar *et al*., 2005). Many authors such as, Jolly *et al*. (2003); Matsushika *et al*. (2008); Ciani *et al*. (2010) reported that many yeast are non-Saccharomyces yeasts like *Candida* sp., *Hansenula*sp., *Kloeckera*sp., *Torulaspora*sp., *Kluyueromyces*sp., *Pachysolentannophilus, Pichia stipites* had the ability to grow and participate in alcoholic fermentation. The ability of yeast to be used in industry depends on many factors such as strains, growth factors and optimum environmental conditions (Negera, 2017). Tolerance of yeast for fermentation product (ethanol-tolerance) and temperature (thermo-tolerance) has great potential to be used in industrial scale fermentation. Isolation and characterization of ethanol and thermo-tolerant yeast from fruits could promote higher yield of ethanol at higher temperature than commercial *Saccharomyces cerevisiae* like baker yeast. Therefore, the main objectives of this study are to isolate and characterize yeast isolates obtained from different fruits and sorghum dough, to evaluate the yeast isolate for ethanol, and heat tolerance for industrial use under different environmental condition.

Methods

Sampling Site and Sample Collection

Fourty different sample of rotten fruits belongs to four species, grapes (*vitisvinifera*), apple (*Malus domestica*), date palm (*Phoenix dactylifera*), banana (*Musa paradisiaca*) and the fifth sample was fermented sorghum dough (Ajeen) which were collected from Atbara town market

in River Nile State northern Sudan, using sterile plastic bags and brought to the Biology Laboratory, Department of Life Science and Environmental Studies – Nile Valley University and the samples were kept at 4 ^{0}C for further study.

Isolation and Enumeration of yeast

One ml of each sample was serially diluted and inoculated onto Yeast Peptone Dextrose Agar (YPDA) medium containing chloramphenicol to avoid bacterial growth and incubated at 37˚C for 3 days. Suspected colonies of yeast were counted as cfu/ml. The selected colonies were re-streaked onYPDA sterile medium (10 g /L Yeast Extract, 20 g/L Peptone, 20 g/L dextrose and 20g/L agar) and the plates were incubated at 37°C for 3 days. Representative colony was picked from the plates and pure cultures were ready for identification procedure as described by AOAD (1998).

Physiological Characteristics

Testing of Yeast Isolates for Carbohydrate Fermentation Using Durham Tube Method

Durham tube method was used for testing the capability of yeasts isolates for carbohydrate fermentation. Yeast fermentation broth with carbohydrate and Durham tube composed of 4.5 g of yeast extracts, 7.5 g of peptone, 80 g of lactose, 120 g of raffinose, 60 g other carbohydrates and 17 g of bromcresol blue per liter of deionized filtered water and final pH 7.1 ± 2 at 25 **^{0}C**. This media (YP broth) was used for characterization of the yeast isolates based on fermentation of specific carbohydrates. The carbohydrates used were glucose, galactose, maltose, sucrose, lactose, fructose and xylose. Yeast fermentation broth media was modified by Wickerham for determination the carbohydrate by detecting the color of the medium and gas formation (Warren and Shadomy, 1991).

Morphological Characterizations

Five yeast isolates were selected for further study and coded as follows, SUDV, SUDA, SUDP, SUDMU and SUDD from grapes (*vitisvinifera*), apple (*Malus domestica*), date palm (*Phoenix dactylifera*), banana (*Musa paradisiaca*) and sorghum dough in order. The vegetative cells morphology determined by growing both in liquid and on solid culture media (YPDA) as described by Kurtzman and Fell, (2006). The macroscopic characteristics were observed such as colony shape, colony edge, colour and texture, while the microscopic features include cell size, shape and methods of vegetative reproduction).

Stress tolerance characterization

Detection of thermo-tolerance

YPD liquid medium was used for detecting thermo-tolerance and growth in liquid media of the selected yeast isolates. The medium was autoclaved at 121°C and 15 psi and cooled. 10 ml portion of the medium was distributed into McCartney tubes, and then inoculated with 48 hours old of the selected yeast isolates. The initial optical density of each tube was recorded on spectrophotometer at 660 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 35°C, 37°C and 40°C for 3 days for observing thermotolerance of yeast isolates. The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer. The treatments were replicated three times and the blank was made of YPD medium without yeast inoculation. The OD is directly proportional to the cell mass or growth (one OD660 nm = 1.85×107cell/ml). The increase in optical density in a flask was recorded as evidence of growth.

Growth in Different pH in Liquid Media

YEPD liquid medium was used for detecting the ability of the selected yeast isolates to grow in different pH, ranged from 4.0 up to10. YPD broth medium was prepared at different pH. Each test-tube contained 13 ml of YPD media with different pH and blank media was used as a control. Then the media were inoculated by half loop full of different yeast cell for each pH then the tubes were incubated at 37°C for 72 hrs. The increase in cell growth was measured using spectrophotometer at 660nm as mentioned above.

Detection of Ethanol Tolerance

For detection of ethanol tolerance, modified YPD broth medium was used as described by Osho, (2005). The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. One ml of various concentrations of absolute ethanol (5% to 25% v/v) was added to different flask of the same medium to constitute varying percentages of ethanol differing by 5% (v/v) from one flask to the others. Forty-milliliter portion of the medium was distributed into 125 ml flask, and then inoculated with selected thermotolerant yeasts. The initial optical density of each flask was read on spectrophotometer at 660 nm against the medium as blank. All cultures were incubated at 37 ^{0}**C** for 3 days and the growth was measured as mentioned above. The increase in optical density in a flask was recorded as growthevidence.

Results and Discussion

Isolation and Screening of Ethanol and Thermo Tolerant Yeasts

A total of thirty yeast isolates were isolated from five different fruit samples namely, grapes (*vitisvinifera*), apple (*Malus domestica*), date palm (*Phoenix dactylifera*), banana (*Musa paradisiaca*) and sorghum dough (Ajeen), collected from Atbara market. Table (1) shows the viable cell count obtained by five yeasts isolates, namely SUDA, SUDV, SUDMU, SUDP and SUDD. The highest colony count was recorded by the isolate SUDD (118 x10^4cfu/ml) while he lowest count was obtained by the isolate SUDV (99 x10^4cfu/ml). The rest of the isolates, SUDA (106 x10^4cfu/ml), SUDMU (112 x10^4cfu /ml) and SUDP (114 x10^4cfu /ml). The various colony counts are indicative of the isolates viability and how actively in industry. In Sudan Sulieman *et al*. (2015) and in Nigeria Umeh and Okafor, (2016) isolated different yeast strains from local sources and they found that the colony forming unit ranged from 3.3× 10^3 -1.6 × 10^6 and 2.7-3.0x 10^5, respectively.

Morphological and Physiological Characteristics of the Yeast Isolates

All the yeast isolates were first screened for carbohydrate fermentation using Durham tube fermentation method in yeast extract peptone dextrose broth. In this study, the yeast isolates showed variation in utilization of seven different sugars (glucose, galactose, sucrose, maltose, fructose, lactose and xylose). Almost all isolates utilized glucose, galactose, sucrose, maltose, fructose and failed to grow on xylose and lactose (Table 2). These results were coincided with that obtained by Kumar *et al*. (2011); Negera (2017) who tested the same above sugar.

Table 1: Viable Cell Count of Yeast Isolates from Different Sources (cfu/ml ×10^{-4})

Yeast Isolates	Sources	Total viable count of different yeast isolates (cfu/ml x10^4)
SUDA	*Malus domestica*	106
SUDV	*vitisvinifera*	99
SUDMU	*Musa paradisiaca*	112
SUDP	*Phoenix dactylifera*	114
SUDD	Sorghum dough	118

Table2: Fermentation of Carbohydrates by Yeast Isolates on Durham Tube Yeast Extract Peptone Dextrose liquid medium fermentation method (YPD)

Isolates	Different Tested Sugars							Total
	Glucose	Galactose	Fructose	Maltose	Sucrose	Lactose	Xylose	
SUDA	+++	++	+++	++	++	-	-	5
SUDV	+++	++	++	+	+++	-	-	5
SUDMU	+++	+++	+++	++	++	-	-	5
SUDP	+++	+++	++	++	+++	-	-	5
SUDDD	+++	++	+++	++	++	-	-	5

Key: + = fermentative, ++ = high fermentative, **+++ =** very high fermentative (empty Durham tube)

in addition to trehalose by six yeast isolates. The same five isolates (SUDA, SUDV, SUDMU, SUDP and SUDD) which recorded high colony forming unit also were relatively high fermentative in Durham tube fermentation method and were selected for further study.

All the selected yeast isolates were observed under compound microscope and cell morphology was observed after 3 days of incubation, at 37 ^{0}C, heavy, dry climbing pellicles were formed on the surface of YPD broth medium. The growth was butyrous with white cream color on YPDA agar (Table 3). The morphological characteristic of all isolates seemed that theyeasts isolates resembled that of *Saccharomyces* spp.as descried by (Kurtzmann, 2006).

Table 3: Morphological Features of Yeast Isolate

Characters	SUDA	SUDV	SUDMU	SUDP	SUDD
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Margin	entire	Entire	Entire	Entire	entire
Color	white	white	Cream, white	white	white
Elevation	Convex	Flat	Convex	Convex	Flat
Cell	spheroidal,ellipsoidal Multilaterial budding	spheroidal, ellipsoidal Multilaterial budding	spheroidal, ellipsoidal Multilaterial budding	spheroidal, ellipsoidal Multilaterial budding	spheroidal, ellipsoidal Multilaterial budding
Pseudomycelium	-	-	-	-	+

Effect of Temperature, pH and Ethanol concentration on the Growth of Yeast Isolates

Different factors such as, temperature, pH and ethanol concentration weretested for optimization the growth of yeast isolates. The effect of temperature on the growth of yeast isolates was study under the range of 25 **^{0}C** -40 **^{0}C**. The highest growth was at 37 **^{0}C** for all isolates and survive up to 40 **^{0}C** (Fig.1). Accordingly, SUDMU recorded the highest growth (0.68 at 660 nm) while the growth of the other isolates ranged from 0.45-0.55nm. In Bangladesh Talukder *et al*. (2016); and

in Ethiopia Negera (2017) found that 37°C was the suitable temperature for the optimum growth of different yeast isolates (Fig.1).

In addition, the growth rate of yeast isolates was measured at different pH values which ranged from 4.0- 10.0 (Fig2). High growth rate wasat pH5.0 and 6.0 for all isolates (Fig.2). Three isolates showed high cell growth at pH6.0, SUDA (1.3) and both SUDP and SUDV were 1.4 while the isolates SUDM and SUDD recorded high cell growth at pH 5.0 (0.72 and 0.6, respectively) Fig.2. The lowest growth was detected at p H 4.0 then pH9.0 and 10.0 while at pH 7.0 and 8.0, the growth rate range was 3.5- 6.0. This result agreed with that obtained by Shamim *et al*. (2016) who found high cell density at pH 5 and 6.

Different ethanol concentrations were added to the broth media (5, 10, 15, 20 and 25%) to test the ethanol tolerance of the yeast isolates (Fig.3). Three isolates showed maximum cell growth at 15% (SUDA, SUDV and SUDD), while the rest of the isolates (SUDMU and SUDP) recorded maximum growth rate at 20% and 25% v/v, respectively. Many studies for ethanol tolerance by yeastisolates was carried out in different countries such as in Nigeria Maxwell *et al*. (2016), isolated twoethanol tolerance yeast isolates, which showed optimum growthat 20% of ethanol concentration while in Thailand Techaparin *et al*. (2017) isolated five ethanol tolerance and their optimum growth was at 13% of ethanol concentration (v/v).

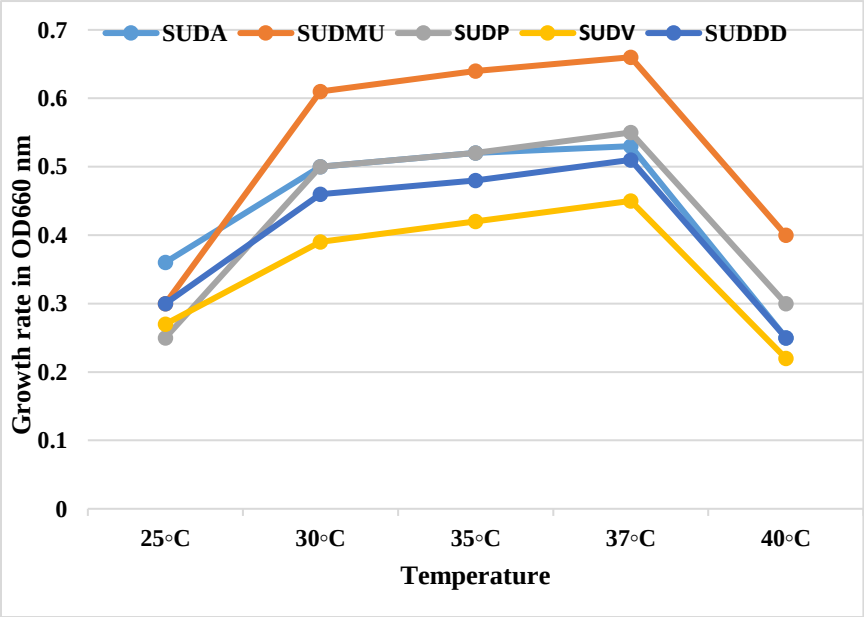

Figure 1: Effect of temperature on the growth of yeast isolates cultivated on YPD broth media

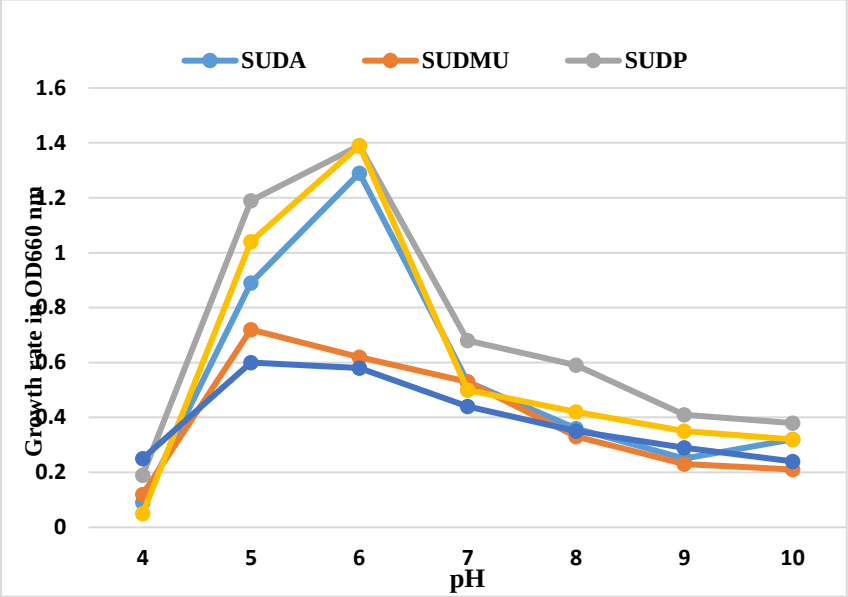

Figure 2: Effect of pH on the growth of yeast isolates cultivated on YPD broth media

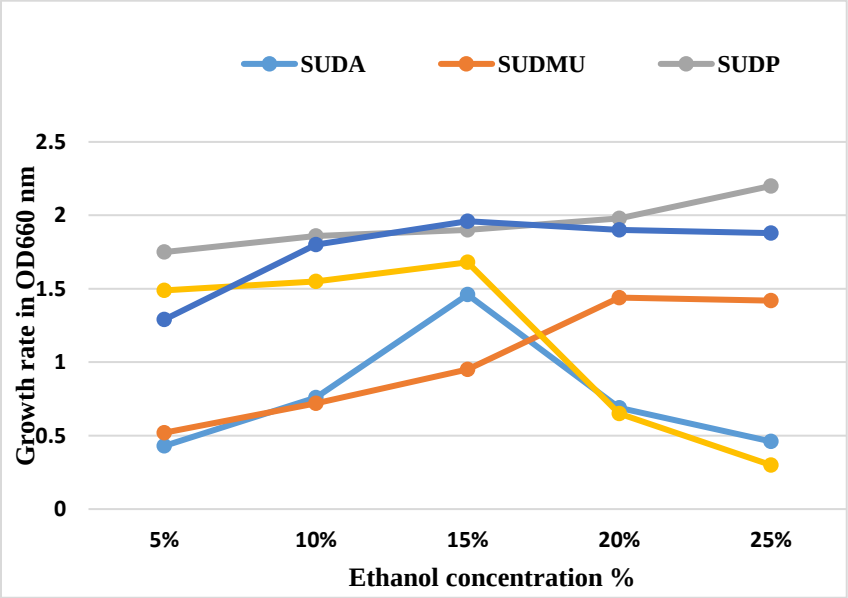

Figure 3: Effect of different concentrations of ethanol on the growth of yeast isolates cultivated on YPD broth media

Conclusion:

Isolation of wild type yeast isolates from local sources is important facet because the isolates are more adapted to the rigors of various environmental stresses of that particular location. In addition, it is an important aspect of the ongoing research for isolation of bioethanol tolerant

yeast isolates. In this study, the best five yeast isolates were identified and selected for further study. According to the morphological and physiological characteristics all the five yeast isolates were resembled that of the genus *saccharomyces*which were closely related to *S. cerevisiae.* All the yeast isolates, showed higher ethanol tolerance at the range 15% - 25% v/v and the mostethanol tolerant yeast isolates were SUDP (25% v/v). The optimum condition for the growth of thefive yeast isolates was showed at 37 ^{0}C and at pH range 5.0 - 6.0.

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