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Bioethanol Production by *Candida tropicalis* Isolated from Sheep Dung

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Abstract:

Microorganisms have an active role in biotechnology for example yeasts, especially in some genus like *Saccharomyces*, *Pichia*, and *Candida*. *C.tropicalis* one of the most important species of *Candida* and despite it is one of the causative agents of candidiasis but it has a major role in the production of many chemical compounds. *C.tropicalis* in the previous study was isolated from sheep dung and morphologically and molecularly classified the result of sequencing was elucidate 100% similarity between the studied isolate and other isolates inserted in DNA Data Bank of Japan DDBJ, physiologically this isolate tolerated 6% ethanol concentration in broth media with the ability to the production of 4% ethanol under optimum conditions including (YEPDB) medium, incubation temperature 30◦C, PH 6 and dextrose as a carbon source which give more productivity as a carbon source and optimum incubation period was three days . This isolate gives a positive result for the cellulase enzyme-producing test and an exciting feature to remove effluent because it completely adsorbed the Congo red dye from dye solation in a liquid growth medium.

Keywords: Bioethanol, *Candida tropicalis*, Cellulase, Dung, Optimum, tolerate.

Introduction:

Bioethanol is the ethanol produced from biological sources such as feedstocks rich in sucrose such as fruit, corn, and sugar cans, lignocellulosic biomass such as wood, micro, and macroalgae biomass by fermenting a wide range of sugars to ethanol [1]. Bioethanol is the most used biofuel worldwide because it reduces crude oil consumption and environmental pollution [2]. Also, microorganisms such as yeasts have an essential role in bioethanol production. There are three essential steps in the generation of bioethanol, the first is an initial test of lignin residues, the second is cellulose and hemicelluloses hydrolysis, and the third is simple sugar fermentation [3, 4]. Mainly most yeast miss the ability of degradation of cellulose to simple sugar so that suggested growing of fungi that have this ability like *Trichoderma reesei* with yeast in the same fermentation medium to produce alcohol [5]. *Candida* is a genus with many species, some found as normal flora in human and animal bodies [6]. *Candida tropicalis* is a common species mainly treated as an opportunistic pathogen, but it is considered a promising tool in biotechnology because of its unique features such as high ability to produce bioethanol, enzymes, and many compounds [7].

The most common yeasts that produce ethanol are *C.tropicalis*, *Saccharomyces pombe*, and *S.cerevisiae*, the first one has fermentation ability, which resembles the last one [8]. One study suggested that the fungus *Trichoderma ressi* can be grown in the same fermentation medium to produce alcohol using cellulose. *C.tropicalis* ferments the starch to ethanol in the little mount, while other strains isolated from herbivore dung can ferments xylose to ethanol with concentrations ranging 1.2-2.3 g/l also produced high concentration of xylitol [9, 10] In cellulose biotechnology, *C.tropicalis* plays an active role in producing extracellular enzymes like cellulose [11]. Also, yeast single cell protein is produced by the same strain of *C.tropicalis* by using sugarcane bagasse hemicellulosic hydrolysate as a substrate of a Carbone source[12].

This study aims to evaluate the ability of *C.tropicalis* isolated from sheep dung to produce ethanol, cellulose enzyme, and adsorption ability.

Material and Methods:

Isolation of Yeast

Sheep dung was collected in a clean paper bag and dried at room temperature. Samples were cultured using moist chamber methods in which sterile filter paper was moistened by sterile water in a petri dish and incubated at 30◦C for three weeks with the periodic examination [13].

Identification of Yeast:

Candida CHROM Agar was used for the morphological identification of Yeast, and micro identification is also carried out by compound microscope depending on [14].

Molecular Identification of Yeast:

The DNA extracted by Presto mini gDNA yeast kit (Bioneer) and amplified using universal primers ITS1: F-5-TCC GTA GGT GAA CCT GCG G- 3 and ITS4: R-5-TCC TCC GCT TAT TGA TAT GC-3. Amplification was carried out by using the EconoTaq plus Green Master Mix (Lucingen). The following PCR program was used: 35 cycles, including an initial denaturation at 95 ◦C for 2 min. Subsequent denaturation was at 95 ◦C for 30 s, annealing at 50 ◦C for 30 s, and extension at 72 ◦C for 1min. A final extension at 72 ◦C for 10min was followed by holding at 4 ◦C. Additionally, ITS Gene space was sequenced by forward, and reverse primers in the Macrogen Inc and the data processed by the BioEdit software and the nucleotide sequence was analyzed using the Basic Local Alignment Search Tool (BLAST). The isolate was inserted in DNA Data Bank of Japan DDBJ and take the name 1FRNOJA and the Accession No is LC647056
http://getentry.ddbj.nig.ac.jp/getentry/na/LC647056

Tolerance and Production of Ethanol

Medium (YPDB)composed of 10gm of yeast extract, 10gm of peptone, and 20gm of dextrose used for producing and tolerance ethanol, different concentration of ethanol prepared (1,2,4,6,8,10)% in broth culture medium and all concentrations are inoculated with isolated *C.tropicalis* after activation. Flasks were incubated for three days at 30◦C. After the incubation period, the absorbance was detected at 600 nm to measure the growth density of Yeast in the medium [15].

Qualitative Determination Test of Ethanol:

The (YPDB) medium was inoculated with an activated isolate of *C.tropicalis* and incubated for three days in 30◦C. After the incubation period, the broth medium of cultivation was centrifuged, and the supernatant was distilled. The reagent (0.5mL sulphuric acid H2SO4and 1mL potassium chromate K2Cr2O7) was added to 1mL of broth medium, and the mixture was incubated at 60°C for 15 min, and volume was completed to 10 ml with distilled water. Transferring the mixture from orange color to blue-green color indicated the presence of ethanol [9].

Quantitatively Determination of Ethanol by Standard Curve

Quantitative estimation is carried out by comparing the absorbance of the medium containing ethanol produced by *C.tropicalis* with different standard curve concentrations in O.D.600 nm [16].

Adsorption of Dye

Dye adsorption ability of *C.tropicalis* cells was studied using Yeast grown in SDB medium until OD reached 1.0 and transferred to congo red solution with a final concentration of 20–100ppm. The experiment was conducted in 100ml conical flasks. Dye absorption was investigated by taking OD 497nm intermittently [9].

Optimization of Ethanol Production

To optimize the production of ethanol by *C.tropicals*, firstly, different mediums were used (potato dextrose broth PDB, sabouraud dextrose broth SDB, yeast extract peptone dextrose broth YEPD). Also, different parameters were tested, including incubation temperature, which was determined by growing the *C.tropicalis* isolate in the most suitable medium at different temperatures (25°, 30°, 37°). Moreover, optimum pH was investigated by cultivating *C.tropicalis* in the medium with pH adjusted to 5.0, 6.0, and 7.0 at the optimum temperature. Likewise, the effect of carbon sources (glucose, maltose, sucrose, and dextrose) was also tested. The incubation period also tested (2,3and 4) days. The growth in different conditions was measured intermittently in OD (optical density at 600 nm) [17].

Cellulase Enzymes Production by *C.tropicalis*

Mandels and Weber medium (MW, 1969), in which carboxymethyl cellulose (CMC) considers as an essential source of carbon and energy, was used to evaluate the ability of *C.tropicalis* to produce cellulase enzyme. Medium composed of (KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, yeast extract, Carboxymethyl cellulose, Agar)
C.tropicalis isolate was grown in MW medium and incubated at 30◦C for three days. The cellulase

activity was achieved by adding a solution of 1% Congo red, which binds to the polymers of cellulose. After 15 minutes, the petri dish was washed with (1M) of NaCl solution, positive test appeared light halo in the zone of yeast colony growth [18].

Biofilm Production Assay:

Congo Red Agar (CRA) medium prepared and active *C.tropicalis* isolate was streaking in medium and incubate at temperature 30◦C 48 hours after incubation period the biofilm-forming isolate was investigated in terms of the appearance of the colony with dry or shiny black appearance while it appeared to isolate not forming biofilms was pink or wine red [19].

Results:

Isolation of Yeast:

After two weeks, many fungi are grown in the dung as well as Yeast.

The yeast isolate is purified on SDA and maintained in slant on 4◦C.

Identification of Yeast Isolate:

The growth of Yeast in SDA was cream-colored and dull smooth colonies after incubating period 3day in 30 ◦C while the color on *Candida* CHROM agar was dark blue colored Fig. 1, A & B, The results of the morphological examination, *Candida* CHROM agar, and sequencing identified the Yeast as *C .tropicalis*. The similarity of nucleotides sequence of this isolate was 100% with other *C.tropicalis* isolates that recorded in DDBJ Fig. 2

Figure 1. Growth of *C. tropicalis* on *Candida* CHROM agar (A) and SDA (B) at 30°C for three days

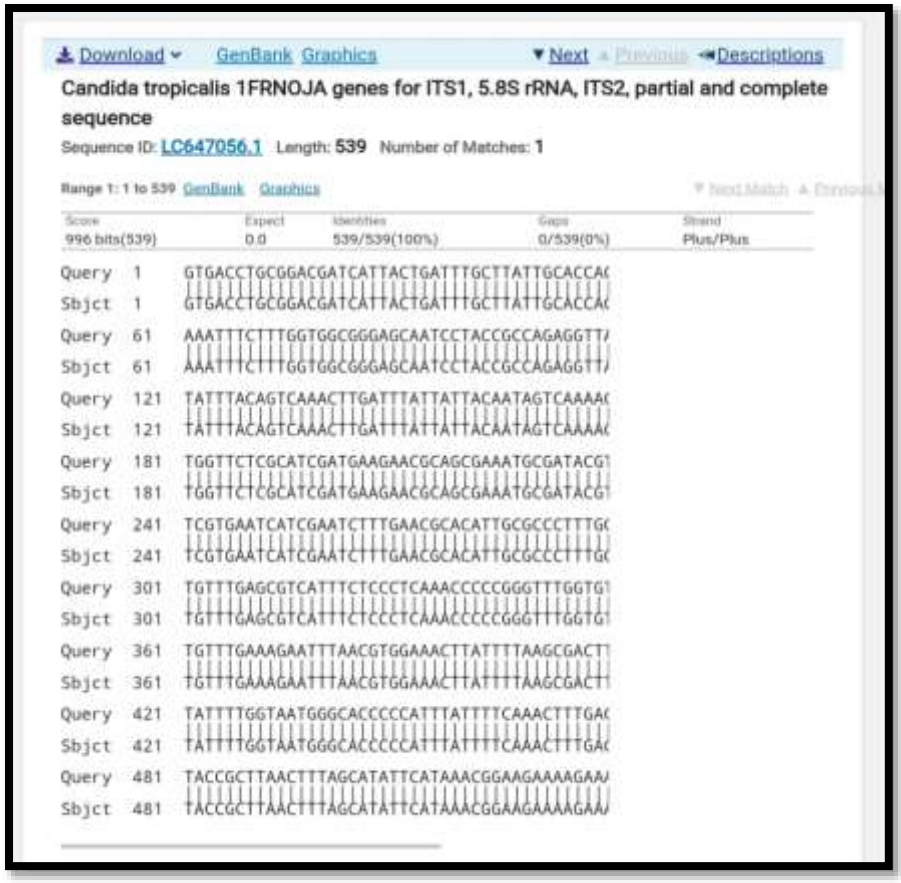

Figure 2. 100% similarity of studied *C.tropicalis* nucleotides sequence with other *C.tropicalis* isolates that recorded in DDBJ.

Tolerance and Production of Ethanol

The isolate *C.tropicalis* tolerated ethanol in the medium it can grow in broth media with 6% ethanol, Table 1.

Table 1. Media absorption with *C. tropicalis* growth at different ethanol concentration after three days at 30°C.

Ethanol concentration (%)	The absorbance of growth media in 600 nm
1	1.2
2	0.9
4	0.5
6	0.2
8	0.0
10	0.0

Qualitative Determination Test of Ethanol :

When potassium dichromate reagent (0.5ml sulphuric acid H2SO4 and 1ml potassium dichromate $K_2Cr_2O_7$) is added to 1ml of broth medium that contains *C.tropicalis* are producing ethanol. The blue-green color indicated that ethanol presence on broth media. Fig. 3.

Figure 3. Detection of ethanol in broth media A (blue-green color = reagent + broth medium with ethanol produced by *C. tropicalis*), B (orange color = reagent + broth medium without growth (control)

Quantitative Determination of Ethanol:

This *C.tropicalis* isolate was found can to produce about 4% V/V of ethanol by comparing the absorption of the ethanol concentration that is found in growing broth medium of *C.tropicalis* in (OD600)with the ethanol standard curve. Fig. 4.

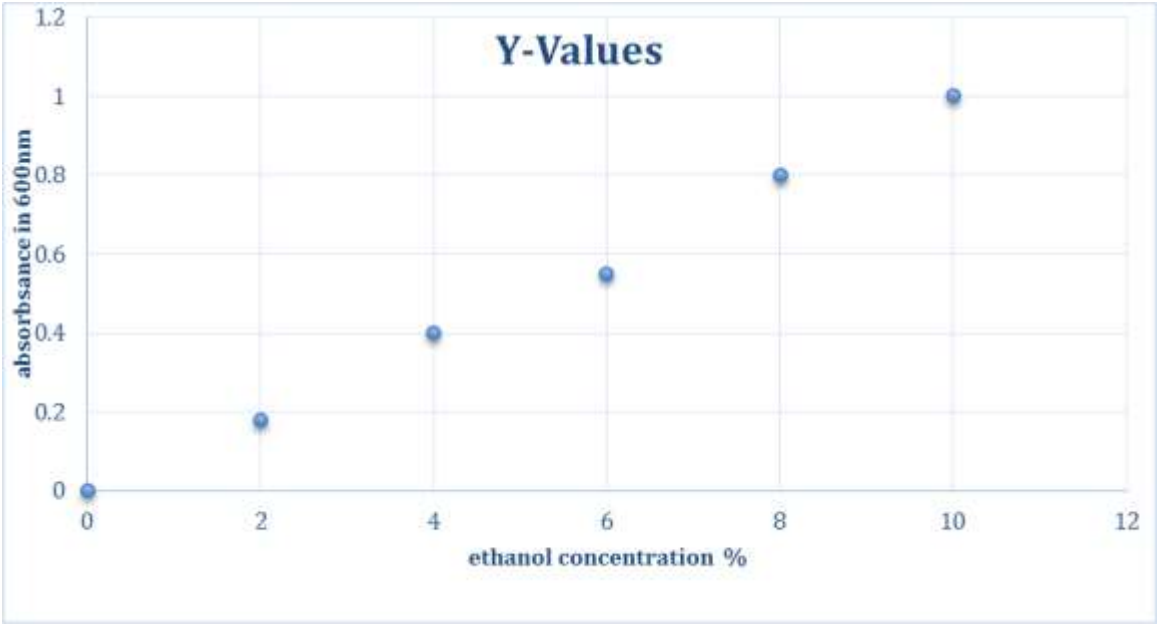

Figure 4. Standard curve of ethanol

Adsorption of Dye

The result showed that the high ability of *C.tropicalis* isolates to adsorb Congo red dye in broth medium after the incubation period, the dye completely removed from the medium in all concentrations of dye . Fig. 5 A&B. In flask A we can see the yeast cells that adsorbed the dye deposited in the flask bottom.

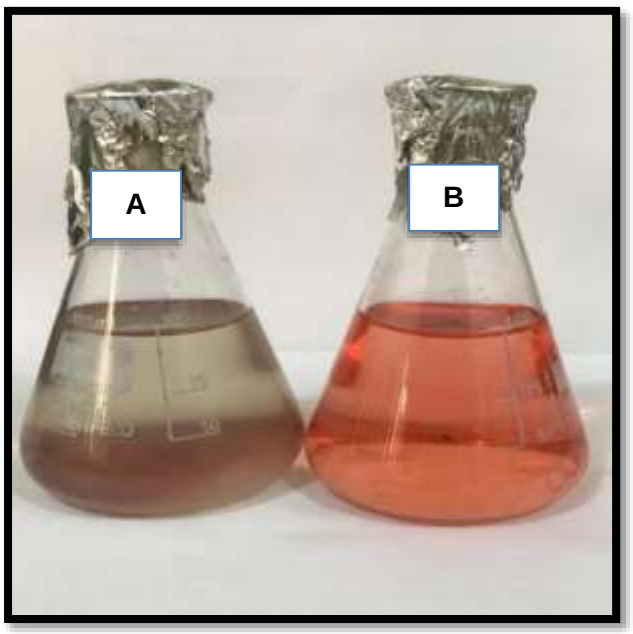

Figure 5. Ability of *C. tropicalis* to adsorb Congo red dye in broth medium, A- after adsorption, B- before adsorption

Optimization of ethanol production:

The result shows that the YEP is the most suitable for producing ethanol at 30° C and PH 6. Dextrose gives more productivity as a carbon source. The optimum incubation period was three days

Cellulase Enzymes Production by *C.tropicalis*:

A clear light halo appeared in the zone of colony growth. This indicates that the CMC in the medium was degraded by the cellulase secreted by *C.tropicalis* colony Fig. 6.

Figure 6. Production of cellulase on CMC medium by *C. tropicalis* after incubation for three days at 30° C appeared as a light halo.

Biofilm Production Assay:

This test revealed that the studied *C.tropicalis* isolate was non-biofilm forming because the isolate appeared with a pink (wine red) colored colony in CRA medium after incubation Fig.7.

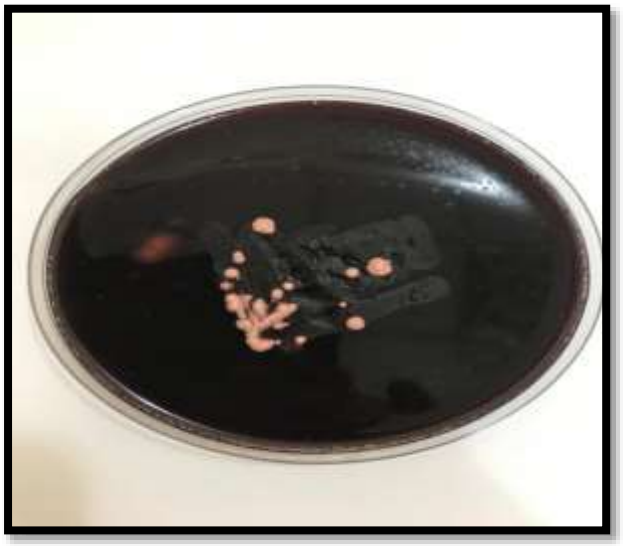

Figure7. Growth of *C. tropicalis* on CRA medium after incubation for three days at 30 °C appeared as pink color colony

Discussion:

Many yeasts capable of fermenting the sugar and starch media supplement alpha-amylase enzyme to ethanol. One of these yeasts is *C.tropicalis* that ferments the hexose sugar to bioethanol [5]. *C.tropicalis* can be isolated from different sources like food samples such as juice and honey [20]. And sugarcane, plant, and garden soil. [5] In a previous study, *C.tropicalis* was isolated from sheep dung-like wise [21] in which many *Candida* species were isolated from herbivores dung with the observation of high ability of these *Candida* isolate to the production of ethanol and xylitol. Initially, the isolate was identified morphologically and molecularly by ITS genetic region because the most widely used molecular targets for identification of yeast are the 28S nuclear ribosomal DNA and internal transcribed spacer region [22]. The studied *C.tropicalis* isolate exhibited tolerance to 6% ethanol concentration in growth broth medium with significant ability to the production of bioethanol with the amount equivalent to 4% (v/v), and this amount was the approach to [7] . in which *C.tropicalis* isolate produced about 5.45% v/v of bioethanol and less than that obtained from *Saccharomyces cerevisiae* RL-11 [23]. Bioethanol C_2H_5OH is either used directly as pure ethanol or mixed with the gasoline to form (gasohol) [2].

In the qualitative assay of ethanol production, the orange color of potassium dichromate reagent transfers to dark green color in the founding of sulfuric acid because of ethanol oxidation to carboxylic acid. The main factor that effecting bioethanol production was culture media. The studying experiment revealed that the optimum medium for ethanol production was YEP with Carbone source Dextrose, and the incubation temperature was 30°C. This is similar to the fermenting process with *C.cerevisiae* that was carried out at 30 °C while the optimum pH was 6 because ethanol production is very high in an acid medium. When pH is raised to 9 the productivity of ethanol will be reduced substantially [2]. The dye adsorption ability of studied *C.tropicalis* isolate was examined by using Congo red dye. The result revealed high adsorption ability and complete removing of Dye from growth broth medium by the yeast cell, which shows this isolate has an exciting feature to remove effluent because adsorption techniques are widely used to remove certain classes of pollutants from water, especially dyes that considers problematic groups because of their ability to pass through conventional treatment system without big change, also some microorganisms like *Chlorella vulgaris* , *Nostoc paludosum* and the yeast like *S.cerevisiae* have dye adsorption ability [24, 25]. The cellulase enzyme test was positive to studied *C.tropicalis* isolate because light clear halo appeared in the zone of colony growth this indicates that the CMC in the medium was degraded by the cellulase secreted by Yeast so that complex carbohydrates (cellulose) turning into simple sugars especially yeast isolated from sheep dung that central it was founded in the medium that cellulose found in the gut of sheep and enzymatic capacity depending on the environment in which the yeasts found this ability to production cellulase enzyme by this *C.tropicalis* isolate consider promising result because the yeast can produced bioethanol from cellulose not only from the fermentation of simple surge also cellulase enzyme play role in the treatment of agriculture waste and the bioremediation of cellulatic materials for sustainable bioethanol production [26, 27] biofilm test show that isolated was non-biofilm forming because the isolate appear with pink (wine red) colored colony in CRA medium after incubation periods, yeast cells in biofilm survive harsh growth conditions as biofilms are surrounded by high molecular weight extracellular polymeric substances (EPS) that attach cells [28,29]. To elimination of biofilms needs amalgamation of hydrolytic enzymes that can degrade proteins, polysaccharides, eDNA, and QS molecules [30]. Cellulase enzyme succeeds in inhibiting biofilm

production in Bacillus sp. by degrading the exopolysaccharide matrix [31]. So that cellulose enzyme prevents biofilm formation in studied *C.tropicalis* because it was isolated from dung that main it was in medium rich with cellulose and cellulase enzyme in the sheep gut.

Conclusion:

C.tropicalis can be isolated from dung as well as this isolate has good productivity to bioethanol and cellulase enzymes so it can be used successfully in production of bioethanol through fermentation of sample sugar and cellulose due to its ability to secrete cellulase enzyme . The high ability of dye adsorption and complete removing of Dye from aqueous solution by the yeast cell will help in decreasing water pollution by removing effluent because adsorption techniques are widely used to remove pollutants from water.

Authors' declaration:

- Conflicts of Interest: None.
- I hereby confirm that all the Figures and Tables in the manuscript are mine. Besides, the Figures and images, which are not mine, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Basrah.

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انتاج الايثانول الحياتي من الخميرة *Candida tropicalis* المعزولة من روث الخراف

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الخلاصة:

تلعب الكائنات المجهرية دور اساسي وفعال في مجال التقنيات الاحيائية و تعتبر الخمائر خير مثال على ذلك خصوصا بعض الاجناس مثل *Saccharomyces, Pichia, and Candida.* من اهم الانواع التابعة للجنس *Candida* فبالرغم من كون هذا النوع *C.tropicalis* احد المسببات الرئيسة لاصابة المبيضات الا انة يتميز بدور كبير في انتاج العديد من المركبات الكيميائية . عزل النوع *C.tropicalis* في هذه الدراسة من روث الاغنام وتم التشخيص باستخدام الطرق المظهرية وكذلك استخدم التشخيص الجزيئي وقد اوضحت نتائج قراءة التتابعات للقواعد النيتروجينة للقطعة الجينية ITS وجود تماثل بنسبة 100% بين العزلة المدروسة والعزلات الاخرى المدرجة في بنك الجينات الياباني كما تم ادراج هذه العزلة في البنك ذاته. اظهرت العزلة قابلية ملحوظة على تحمل الايثانول في الوسط الزرعي حيث تمكنت من تحمل تركيز 6% من الايثانول في الوسط الزرعي , وتمكنت من انتاج الكحول الاثيلي في الوسط الزرعي السائل بنسبة 4% تحت الظروف المثلى المتمثلة بالوسط YEPDB وسط خلاصة الخميرة مع الببتون والدكستروز ودرجة حرارة الحضن المثلى كانت 30 درجة سليزية ودالة حامضية 6 كما عد الدكستروز المصدر الكاربوني المثالي الذي اعطت فيه الخميرة اعلى انتاجية للايثانول وفترة الحضن المثلى كانت ثلاثة ايام. وتميزت العزلة كذلك من انتاج انزيم السليليز حيث كانت نتيجة النمو على وسط CMC كاربوكسي مثيل سليلوز ظهور هالة شفافة في موقع نمو الخميرة تشير الى تحلل السليلوز في منطقة الهالة نتيجة افراز الانزيم المحلل للسليلوز كما تميزت هذه العزلة بقابلية عالية على امتصاص الصبغات الكيميائية من الاوساط السائلة مما يفتح افاق جديدة في مجال تنقية المياه من الملوثات. هدف لدراسة هو انتاج الايثانول الحياتي , وانزيم السليلز من الخميرة *C. tropicalis* وتقييم امكانية استخدام هذه الخميرة في ازالة الملوثات من البيئة المائية.

الكلمات المفتاحية: الايثانول الحيوي , *Candida tropicalis*, سليليز , روث , الظروف المثلى ,تحمل .