EVALUATION OF THE CELLULYTIC FUNGI ISOLATED FROM AGRICULTURAL WASTES PRODUCING OF BIOETHANOL

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ABSTRACT:

The number of fungal isolated in this research were 48 fungal isolates that isolated from plant, agricultural and animal waste from three different regions outside Baghdad, and three different regions in Baghdad, screening 24 cellulolytic isolates fungi, which were obtained 8 isolated fungi *Aspergillus parasiticus, Trichoderma harzanium, Trichoderma viride, Chladosporium ssp, Alternaria ssp,* mycelia asterilia (1), *Metarhizium spp* and mycelia asterilia (2). Capable of producing bio-alcohol with simultaneous saccharification and fermentation without using the yeasts in fermentation methods isolates which recorded the following; 13.547, 10.511, 8.298, 7.951, 6.668, 6.597, 2.455 and 1.256 ppm. respectively. The best optimal conditions for growth were 30ºC and pH 7.

Key word: bioethanol ,fungi, fermentation , rice straw

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تقييم الفطريات المحللة للسيليلوز المعزولة من المخلفات الزراعية، النباتية والحيوانية المنتجة للإيثانول

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المستخلص

بلغ عدد الفطريات المعزولة في هذا البحث 48 عزلة فطرية من مخلفات نباتية وزراعية وحيوانية من ثلاث مناطق خارج مدينة بغداد وثلاث مناطق اخرى داخل المدينة وتم انتخاب 24عزلة فطرية محللة للسيليلوز ومنها تم الحصول على 8 عزلات فطرية *Chladosporium ssp* ، *Trichoderma viride*، *Trichoderma harzanium*، *Aspergillus parasiticus*، *Alternaria ssp*، mycelia asterilia (1) ، *Metarhizium sp* و mycelia asterilia (2) قادرة على انتاج الكحول الحيوي بواسطة التحلل السكري والتخمير لقش الارز بعملية واحدة دون استخدام الخمائر في عملية التخمير حيث سجلت 13.547،10.511، 8.298 ، 7.951، 6.668، 6.597، 2.455 و 1.256 جزء بالمليون لكل منها على التوالي. وكانت الظروف المثلى للنمو عند درجة حرارة 30 م° ودالة حامضية 7 .

الكلمات المفتاحية: الكحول الحيوي، الفطريات، التخمير، قش الارز.

البحث مستل من رسالة ماجستير للباحث الرابع.

INTRODUCTION:

Fungi play important role as a biocatalysts for the production of food, chemicals and fuels. They are becoming important in the devel - opment of solar energy technology, biodegr - adation and bioremediation (1). Fungi are ch - emotrophs, obtaining energy from chemicals, and heterotrophs, using organic compounds as carbon source (2). They secrete extracellular enzymes to degrade polymers outside the cell and absorb the released nutrients and transport water through the cell membrane (3). Most of fungi that grow on dead trees (saprotrophic fungi), and their role in natural ecosystem is by decomposition of wood and recycle the nutrients of wood. Wood degrading fungi are further divided into white-rot and brown-rot fungi. Wood decay mechanisms of both groups rely on enzymes (radical formation), low pH, and the production of organic acids (4). Plant materials are composed of three major units, cellulose, hemicellulose and lignin. Lignocellulosic materials including agricultural wastes, forestry residues, grasses and woody materials have great potential for biofuel production. Agricultural lignoce - llulosic biomass is comprised of about 10–25% lignin, 20–30% hemicellulose, and 40–50% cellulose (5). Cellulose is the most common organic renewable resource on earth **(**6). Conversion of cellulose into glucose consists of two steps via enzyme system of fungi. In the first step, beta-1, 4 glucanase breaks the glucosidic linkage to cellobiose, and then beta-1, 4 glucosidic linkages is broken by beta-glucosidase (7). Fungi are hetrerotrophic organism depending on carbon compounds synthesized by other living organism (8). Fungi may have a significant role in commercial biofuel production, by degrading lignocellulose material and many of them are good producers of solvents. A number of filamentous fungi have been evaluated for their ability to produce biofuels like *Trichoderma spp.* which is a cellulose-degrading isolated from soil fungus and decaying plant material throughout the world, and produce fermentable sugars easily then converted to ethanol **(9).** The aim of this research is to evaluated production of bioethanol from the rice straw available in Iraq as an energy source using microorganisms (fungus).

Materials and methods:

Samples collection

The number of samples collected from agricultural, plants and animals wastes from different regions in Baghdad city were 134 samples.

Isolation and identification of fungi:

A serial dilution technique was used for isolation of fungi according to the Waksman (10). Immediately, each suspension was serially diluted and transferred into plates with potato dextrose agar media, Roe Bengal agar and malt extract agar according to the Nazir *et al.,* (11). The isolated fungi was identified according to the Samson and Hockstra (12) and the Pitt *et al.,* (13) identification.

Growth capability (qualitative evaluation)

The fungi were placed on two plates of Czapek Dox agar supplemented with carboxymethyl cellulose (1.2% w/v) and cellulose (0.6 w/v).The czapex dox agar was prepared - ccordingtothe Devi (14**)** procedure. After an appropriate incubation period of 5-7 days growth of fungi and cellulolytic activity was detected by appearance of clear zone around the colonies. Hydrolytic zones around the growing colonies were recorded for carboxymethyl cellulose activity. To enhance the visibility of hydrolytic zones, the plates were treated according to the Gomashe **(**15) method.

Optimum conditions for growth of cellulytic fungi (pH, incubation period and temperature)

Selected isolate was grown in modified Czapex Dox medium 1% CMC and 0.6% cellulose by stabbing on petri dish culture media from spore suspension and pH adjusted by using 0.1N HCl and 0.1N NaOH to (5.8 and 7.8) and incubated at different temperatures: 27, 30 and 34° C for 3-7days.

Pre-treatment of rice straw

Rice straw was washed, air dried, size fractioned to 0.5mm via grander. About 50 g milled dried rice straw were suspended in 5% NaOH in ratio of 1: 10 (w/v) rice straw : NaOH. After that the samples were boiled in water bath 85ºC for 1 h. Finally, pretreated sample was pressed through cheese cloth. The

pellet was washed with distilled water to remove NaOH, dried at 60 C° overnight to constant weight, and stored at room temperature for further use (**16**).

Ethanol production by liquid media

The cellulose and hemicellulose fraction of rice straw can be converted to ethanol by either simultaneous saccharification and fermentation. The Cellulolysis Basal Medium (CBM) was supplemented with 4 gm of Alkali pre-treatment rice straw milled rice straw, and then inserted into each 100 ml Erlenmeyer flask with cotton stopper. After autoclaving, it was inoculated with 5 ml from fungal spores suspension containing 10^6 spores/mL^{-1}. The cultures were incubated at 150 rpm and 30 °C for 14 days. After 14 days of cultivation, culture aliquots were filtered through cheese cloth (gauze) and centrifuged at 5000 rpm to remove solids and filtered using sterile membrane filter (0.2 mm), distilled alcohol fractional under vacum by the evaporator device Rotary. Then alcohol was separated from the rest of contaminate organic by ethyl acetate volume to volume 2:2 ml, and the produced ethanol was measured by using gas chromotography (GC) system **(16).**

Result and Discussion

Characterization of isolated fungi

From samples, 48 isolates of fungi (18 genera) Mucor spp., Aspergillusspp ,Metarhizium spp,Cladosporium spp, Penicillium spp, Fusarium spp, Emericella , Yeast colonies, Alternaria spp., Bipolarisspp, Trichoderma spp, Rhizopus spp, Rodotorula, Cleistothecium spp, Geotrichum candidum ,Scopularopsis spp. ,mycelia sterilia and Aerobasidium spp.from samples from agricultural, plants and animals waste were isolated. The fungus isolates were differed accordingly to different places and varieties.

Screening of Cellulolytic isolated fungi

According to previous studies the screening of 48 fungal isolates are (qualitative evaluation and cellulytic activity) The results indicated that only 24 isolates were selected as they represented the highest ability to cellulolytic; namely AO, C1, TH1, AN1, R1, TV, PG, AF, B1, L1, AP, TH, AP1, AN3, AO2, A, A1, C, F, AO1, C2, F1, CL and AP2.

Determinasion the optimum pH and temperature

Acid degree has a direct effective on growth activity through metabolism of fungi. The results of three levels of pH 5.8, 7, 7.8 were affective studies in fungi growth on medium Czapek Dox of agar media supplemented with CMC (1.2% w/v) , Czapek Dox agar media supplemented cellulose(0.6 w/v) as shown in Table 1. The optimum pH,7 has a good susceptibility for growth of fungal isolates, excepting the fungi isolates Y, PG1, B2, CM, R, PV and B2 which are not growing in this rate. The lower rate of growth was shown in the pH, 5.8. The effect of pH on growth of fungi isolates was caused through the salts solubility in medium and ionization of nutrition were transported; thus acilitating the developing microorganism (17).Temperature affects in the identification activity of various microorganisms including fungi especially the growth and metabolism. It is a fundamental way to control all activities catabolism and metabolism of these microorganisms (18**).** In order to investigate the role of temperature on development and growth of the fungus, the results of three different incubation temperatures were used 27, 30, and 34 C° studies in fungi growth on medium of Czapek Dox agar media supplemented with carbox - ymethyl cellulose (1.2% w/v) , Czapek Dox agar media supplemented cellulose(0.6 w/v)) as shown in Table 2. The optimum temperature 30 C° has a good susceptibility for the growth of fungal isolates; lower level of growth was obtained at 34 C°.The fungi isolates Y, PG1, B2 and R are not growing in rate of temperatures.

Ability of ethanol production

The results in Table 3 showed 8 fungi isolates that have the ability of bioalcohol production from 24 tested fungi isolates; namely TH1, TH, TV, AP, A1, CL, LI and C. The AP isolate recorded a highest product, 13.547 p.p.m. whereas the TH isolate recorded the second degree 10.511 p.p.m. while the isolate CL gave lower bioalcohol product reaching 1.256 p.p.m. The bioethanol production from lyses the cellulose and hemicelluloses in rice straw to simplest fractions and converted into ethanol production by simultaneous saccharification and ferm- entation without using the yeasts in fermentation methods. Therefore, this fungi isolate gave a good

alternative isolates instead of the *Sacchar - omyces cerevisiae* in ethanol production. These results did not agree with Muryanto *et al.*,(19), studies who succeeded in converting lingo- celluloses and particularly oil palm empty fruit bunch to bioethanol by sold state fermentation-ssf process, *Rhizopus oryzae* are able to produce ethanol in the high yield. Besides, Parameswaran *et al.*,(20) study showed the ability of fungi to convert the lignocelluloses in rice straw to sugar and then to bioalcohol by simultaneous saccharification and fermentation without using yeast. These results show efficient performance than the results of other researchers (19,20).

Table 1. The optimum pH effective on fungi isolate growth on different media

Abbr.	Name of genus	Cmc			Cellulose		
		5.8	7	7.8	5.8	7	7.8
AO	*Aspergillus ochraceus*	19 +	38 ++	26 ++	5 +	30 +	24 +
C1	*Chladsporium 9*	14 ++	25 +++	22 +++	3 +	16 +++	13 +++
GC	*Giotricum candidum*	-	54 ++	52 ++	-	22 +	18 +
TH1	*Metarhizium spp. 15*	69 ++	80 +++	80 +++	54 ++	80 ++	80 ++
AN1	*Aspergillus niger 3*	15 +	46 +	41 +	32 +	72 +	70 +
R1	*Clestothecium spp.9*	36 ++	68 ++++	62 +++	26 +	56 +	53 +
TV	*Trichoderma viride*	72 +++	80 +++	80 +++	74 ++	80 ++	80 ++
PG	*Penicillium glabrum*	-	26 ++	23 ++	-	33 +	26 +
AF	*Aspergillus flavus 4*	24 ++	54 +++	51 +++	16 +	46 +	43 +
MH	*Mucor hemalis 10*	47 ++	80 ++	76 ++	34 +	80 +	78 +
RO	*Rhizopus oryzae 13*	35 ++	80 ++	74 ++	33 +	33 +	28 +
RS	*Rhizopus stolonifer 7*	9 +	80 ++	75 ++	-	44 ++	40 ++
AT	*Aspergillus terreus 13*	28 ++	40 ++	36 ++	30 +	30 +	25 +
B1	*Bipolaris spp 9*	43 +++	56 +++	53 +++	16 ++	36 ++	31 ++
S1	*Scopularopsis spp*	11++	22 ++	20 ++	-	-	-
E1	*Emercella spp 22*	22 +	44 +++	41 +++	12 +	32 ++	28 ++
L1	*Mycelia stereilia 8*	26 ++	34 ++++	32 ++++	16 +	26 ++	24 ++
Y	*Yeastes*	-	-	-	-	-	-
PG1	*Penicillium glabrum 3*	-	-	-	-	-	-
AP	*Arobasidium pullulas 21*	18 ++	26 ++	24 ++	-	-	-
B2	*Bipolaris spp.*	-	-	-	-	-	-
M	*Mucor spp. 11*	45 ++	78 +++	74 +++	35 +	65 ++	62 ++
CM	*Chlado. marcrcum*	-	-	-	-	-	-
R	*Rhodotorula 5*	-	-	-	-	-	-
TH	*Trichoderma harazinum*	55 ++	80 +++	76 +++	55 ++	65 ++	61 ++
AN2	*Aspergillus niger 15*	34 +	80 +	76 +	-	-	-
FS	*F. sporotrichiodes15*	24 ++	42 +++	37 ++	13 ++	34 ++	31 ++
AP1	*Aspergillus parasiticus*	33 ++	33 ++++	30 ++++	11 +	12 +	8 +
AN3	*Aspergillus niger 6*	9 ++	30 ++	24 ++	-	3 +	3 +
E	*Emericella spp. 18*	-	11 +++	9 +++	-	18 ++	12 ++
AO2	*Aspergillus ochraceus 20*	13 ++	23 +++	20 +++	6 +	16 ++	13 ++
A	*Alternaria alternate 19*	19 +++	40 +++	36 +++	7 +	33 ++	27 ++
M2	*Mucor spp. 16*	46 ++	76 ++	74 ++	25 +	60 +	55 +
A1	*Alternaria spp.20*	11 ++	51 +++	47 +++	2 +	22 ++	16 ++
C	*Chladspoirium spp.19*	5 ++	20 +++	17 +++	-	5 ++	4 ++
F	*Fusarium spp.12*	30 ++	60 +++	53 +++	11 +	32 +++	29 ++
AO1	*Aspergillus ochracus 12*	4 ++	14 +++	11 +++	-	8 +	5 +
P	*Penicillium spp.18*	-	27 +++	24 +++	-	11 ++	8 ++
PG2	*Pencillium glabrum 4*	-	10 ++	7 ++	-	10 ++	6 ++
C2	*Chladsporium spp.20*	2 ++	17 +++	15 +++	-	11 ++	8 ++
F1	*Fusarium spp 22*	53 +	73 +	67 +	46 +	60 +	56 +
CL	*Mycelia sterilia 20*	20 ++	40 +++	35 +++	23 +	33++	30 ++
F2	*Fusarium spp. 19*	57 ++	80 +++	75 +++	45 +	80 ++	76 ++
E2	*Emercella spp. 20*	45 +	55 ++	52 ++	24 +	43 +	38 +
PV	*Penicillium variable 24*	-	-	-	-	-	-
AP2	*Asp. parasiticus 23*	6 +	14 +++	11 +++	3 +	10 +	7 +
AT1	*Aspergillus terruse*	11 +	30 +	23 +	-	-	-
AN4	*Aspergillus niger 21*	3 ++	28 ++	24 ++	-	10 ++	7 ++

- No growth, + Poor growth, ++ Moderate growth, +++ Good growth, ++++ Massive growth

Table 2. Ssusceptibility growth of isolates on CMC and cellulose size in mm.

Abbr	Name of genus	Cmc			Cellulose		
		27C°	30°C	34C°	27C°	30C°	34C°
AO	*Aspergillus ochraceus*	31 +	38 + +	18 +	27 +	30 +	21+
C1	*Chladsporium 9*	22+ +	25+ + +	16 +	11+ +	16+ + +	9 +
GC	*Giotricum candidum*	50+ +	54+ +	49 +	18 +	22+	16 +
TH1	*Metarhizium spp. 15*	76 + +	80+ + +	71 ++	76+ +	80+ +	75+ +
AN1	*Aspergillus niger 3*	44+	46+	43+	62+	72+	54+
R1	*Clestothecium spp.9*	67+++	68++++	60++	53+	56+	51+
TV	*Trichoderma viride*	80++	80+++	78++	78+	80++	77+
PG	*Penicillium glabrum*	25++	26++	21+	30+	33+	23+
AF	*Aspergillus flavus 4*	52++	54+++	52+	38+	46+	34+
MH	*Mucor hemalis 10*	80++	80++	80+	76+	80+	65+
RO	*Rhizopus oryzae 13*	78+ +	80++	73+	29+	33+	22+
RS	*Rhizopus stolonifer 7*	76++	80++	79+	40+	44++	38+
AT	*Aspergillus terreus 13*	37++	40++	38++	24+	30+	22+
B1	*Bipolaris spp 9*	55++	56+++	51+	33+	36++	31+
S1	*Scopularopsis spp*	23+	22++	22+	-ve	-ve	-ve
E1	*Emercella spp 22*	40+++	44+++	32+	26+	32++	25+
L1	*Mycelia stereilia 8*	31+++	34++++	25+++	23+	26++	21+
Y	*Yeastes*	-ve	-ve	-ve	-ve	-ve	-ve
PG1	*Penicillium glabrum 3*	-ve	-ve	-ve	-ve	-ve	-ve
AP	*Arobasidium pullulas 21*	23+	26++	22+	-ve	-ve	-ve
B2	*Bipolaris spp.*	-ve	-ve	-ve	-ve	-ve	-ve
M	*Mucor spp. 11*	79++	78+++	72+	55+	65++	46+
CM	*Chlado. marcrcum*	2+	-ve	-ve	-ve	-ve	-ve
R	*Rhodotorula 5*	-ve	-ve	-ve	-ve	-ve	-ve
TH	*Trichoderma harazinum*	80++	80+++	76+	62+	65++	62+
AN2	*Aspergillus niger 15*	64+	80+	33+	-ve	-ve	-ve
FS	*F. sporotrichiodes15*	40+++	42+++	32+	31+	34++	28+
AP1	*Aspergillus parasiticus*	33++	33++++	31++	10+	12+	7+
AN3	*Aspergillus niger 6*	30+	30++	24+	-ve	3+	-ve
E	*Emericella spp. 18*	9++	11+++	7+	11+	18++	-ve
AO2	*Aspergillus ochraceus 20*	22++	23+++	20+	12+	16++	7+
A	*Alternaria alternate 19*	37+++	40+++	36++	28+	33++	26+
M2	*Mucor spp. 16*	74++	76++	71+	55+	60+	54+
A1	*Alternaria spp.20*	43++	51+++	40++	20+	22++	19+
C	*Chladspoirium spp.19*	18+	20+++	16+	3+	5++	-ve
F	*Fusarium spp.12*	56++	60+++	44+	27+	32+++	30+
AO1	*Aspergillus ochracus 12*	12++	14+++	11+	8+	8+	4+
P	*Penicillium spp.18*	18++	27+++	21+	9+	11++	4+
PG2	*Pencillium glabrum 4*	8++	10++	-ve	6+	10++	-ve
C2	*Chladsporium spp.20*	2+	17+++	10+	9++	11 ++	8 ++
F1	*Fusarium spp 22*	54+	73+	56+	54+	60+	48+
CL	*Mycelia sterilia 20*	35++	40+++	32+	32+	33++	28+
F2	*Fusarium spp. 19*	74++	80+++	70+	66+	80++	64+
E2	*Emercella spp. 20*	54+	55++	41+	37+	43+	33+
PV	*Penicillium variable 24*	12+	-ve	-ve	7+	-ve	-ve
AP2	*Asp. parasiticus 23*	11++	14+++	9+	7+	10+	6+
AT1	*Aspergillus terruse*	23+	30+	22+	18+	-ve	-ve
AN4	*Aspergillus niger 21*	24+	28++	20+	8+	10++	5 +

- No growth, + Poor growth, ++ Moderate growth, +++ Good growth, ++++ Massive growth.

Table 3. Activity of ethanol production

Fungus	Abb.	Cons. ppm	Fungus	Abb.	Cons. Ppm
Mycelia sterilia 20	**CL**	**1.256**	***Trichoderma viride***	**TV**	**8.298**
Aspergillus ochraceus	**AO**	**0.00075**	***Aspergillus niger 3***	**AN1**	**0.005**
Alternaria spp.20	**A1**	**6.668**	***Chladsporium 9***	**C1**	**0.0003**
Penicillium glabrum	**PG**	**0.0002**	***Clestothecium spp.9***	**R1**	**0.012**
Trichoderma harazinum	**TH**	**10.511**	***Mycelia stereilia 8***	**LI**	**6.597**
Metarhizium spp. 15	**TH1**	**2.455**	***Arobasidium pullulas 21***	**AP**	**0.00012**
Aspergillus parasiticus	**AP1**	**13.547**	***Chladspoirium spp.19***	**C**	**7.951**
Aspergillus niger 6	**AN3**	**-ve**	***Aspergillus flavus 4***	**AF**	**-ve**
Alternaria alternate 19	**A**	**-ve**	***Fusarium spp.12***	**F1**	**-ve**
Aspergillus ochraceus 20	**AO2**	**-ve**	***Bipolaris spp 9***	**B1**	**-ve**
Chladsporium spp.20	**C2**	**-ve**	***Asp. parasiticus 23***	**AP2**	**-ve**
Fusarium spp.12	**F**	**-ve**	***Aspergillus ochracus 12***	**AO1**	**-ve**

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