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RESEARCH ARTICLE

BIOETHANOL PRODUCTION FROM FRESH ONION RESIDUES BY YEAST

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ABSTRACT

Egypt is one of the largest countries of onion production; thus, possibility of ethanol production from onion residues by fermentation process was investigated. Onion residues of some Egyptian cultivars, including Giza 6, Giza 20, Giza red and Shandaweel1, were used for ethanol production by *Saccharomyces cerevisiae* as a biocatylast under different parameters, such as temperature, pH and using *Botrytis cinerea* as biodegrading bioagent. The results showed that the total sugars content of plants juice was 43.14 for Giza 6, 36.0 for Giza 20, 41.5 for Giza red, and 32.1 g/L for Shandaweel1. The optimal fermentation conditions for ethanol production from onions juice were found to be 6.0 for pH and 30 °C for temperature. Under these conditions highest ethanol production rate of 86% (6.4 g\L\hour) was obtained from Giza 6 cultivar residues treated with *B. cinerea*.. Based on these results, onion residues which is an agricultural waste is a promising alternative source for bioethanol production by *S. cerevisiae*.

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INTRODUCTION

Plant biomass is one of the most important and available sources rich with different substrates which could be converted by microorganisms to biofuels, such as bioethanol on industrial scale (Antoni *et al.*, 2007). However, in addition to the complicated processes, there are several crises are present during fossil fuel production; therefore, many researchers attempt to find safe and cost-effective fossil fuels alternatives (Sharma *et al.*, 2008). Biomass has been the cleanest and predominant alternative of ethanol fuel (Lin and Tanaka, 2006). The cars started to use ethanol as fuel source until 1930s, but because the low cost supply of fuel from petroleum and natural gas, the interest in liquid fuel of biomass was decreased (Jain and Sharma, 2011). Due to the limited fossil oil supplies expectations, the focus on the need for alternative fuel renewed. Using microorganisms for ethanol production through sugar fermentation , starche or cellulose is a target for many scientists at the the present time (Lin and Tanaka, 2006).

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Saccharomyces cerevisiae is important microorganism for ethanol production process (Jain and Sharma, 2011).

Onion is one of vast production vegetables in Egypt specially Giza 6 cultivar (Hussein *et al.*, 2014). Onion production is one of most used and consumed vegetables all over the world (FAOSTAT, 2013). Even fermented ethanol from onion can be used as a substrate in steps such acetic fermentation for onion vinegar (Horiuchi *et al.*, 1999). The total sugar contents of onion, vitamins, and minerals, is represent an ideal source for ethanol production using microorganisms and favorable source for ethanol fermentation. (Ghabel *et al.*, 2010). Using the residues of onion plants by repeated batch process as continuous process is the most suitable process for production of ethanol (Vazirzadeh *et al.*, 2012). Repeated batch process is easy and stable process of ethanol production (Robati, 2013). Economic value appears by producing ethanol biofuel on long term (Bothast and Schlicher, 2005). In this study, we used different onion cultivars redidues for bioethanol production by *S. cerevisiae* at different treatment including pH's, temperatures, and pretreatment by biodegradation of onion residues by *Botrytis cinerea* and by steam explosion were also studied.

MATERIALS AND METHODS

Microorganisms and culture conditions

Botrytis cinerea was provided from Assiut University Mycological Centre (AUMC) culture collection, Assiut University, Assiut, Egypt, and maintained on potato dextrose agar (PDA) medium revived for at 25±2 °C for 7 days then stored at 4 °C. *Saccharomyces cerevisiae* was provided from Assiut University Mycological Centre (AUMC) culture collection. Yeast Potato Dextrose (YPD) medium containing 1g glucose/dextrose, 1 g yeast extract, and 2 g peptone per 100 ml distilled water, was used for the inoculums. Onion cultivars residues of Giza 6, Giza 20, Giza red and Shandaweel1 were used as a substrate for ethanol fermentation, were collected from different localities in Assiut governorate, Egypt. Onions residues cut and pressed in a mechanical mixer.

Cellulase production activity

Detection of exo-1.4 ß-glucanase (C1)

The cellulase production from *B. cenerea* was screened for their abilities to produce exo-1, 4ß-glucanase (C1). The tested fungi were inoculated first into 250 ml Erlenmeyer's conical flasks containing 50 ml of cellulase production medium which has the composition (g/l) $(NH4)_2SO_4$ 0.5, L-asparagine 0.5, KH_2PO_4 1.0, KCL 0.5, $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.2, Yeast extract 0.5 and cellulose microcrystalline 10, as described by Luo *et al.* (1997). The pH was adjusted to 7.0 using acetate buffer and incubated at 28º ±1°C for 7 days under static conditions. The growth was filtrated using filter paper Whatman No 1, the filtrate considered as a crude enzyme. After incubation period, a well in the agar medium (has the same composition as above) was filled with 100 µl of the filtrated crude enzyme, and the plates were incubated at 28°C for 24hr.; after incubation the plates were flooded with 1% iodine solution (1 g iodine + 3 g KI + 100 ml water) and shaken for five minutes, the iodine solution was poured of from the plates. Clear zone around wells indicates hydrolysis of cellulose by the releasing exo-1, 4-β-glucanase (C1). The activity of enzyme was expressed as (mm), by measuring the clear zone around the wells.

Detection of endo-1.4 ß-glucanase (Cx)

The cellulase production from *B. cenerea* was screened for their abilities to produce endo-1, 4 ß-glucanase (Cx). The tested fungi were inoculated into 250 ml Erlenmeyer's conical flasks containing 50 ml of Oksanen medium (Oksanen *et al.*, 2000), which containing (g/l): $(NH_4)_2SO_4$, 2.1 g carboxymethyl cellulose (CMC) 10 g, KH_2PO_4 1.0 g, $MgSO_4.7H_2O$, 0.5, and agar, 15g. The pH was adjusted to 7.0 using acetate buffer and the inoculated flasks were incubated at 28ºC ±1°C for 7 days under static conditions.

The mycelial growth was filtrated using filter paper Whatman No 1, the filtrate considered as a crude enzyme. After incubation a well made by using cork borer in the agar medium of the same composition as above was filled with 100 µl of the filtrated crude enzyme and the plates were incubated at 28°C for 24 hr.; after which the plates were flooded with 1% iodine solution (1 g iodine+ 3 g KI+ 100 ml water) and shaken for five minutes, the iodine solution was poured of from the plates. Clear zone around wells indicates hydrolysis of cellulose by the releasing endo-1, 4-β-glucanase (Cx). The activity of enzyme was expressed as (mm), by measuring the clear zone around the wells.

Pectinase production activity

The method was carried out as described by (Hankin *et al.*, 1971). The used medium contained the following constituents which were prepared in two main portion (g/L):

Portion A: Yeast extracts 1g, Pectin from citrus peel 5g, Agar 15g, distilled water 500 ml, and its pH was adjusted to 7.0.
Portion B: Mineral salt solution composed (per liter) of : $(NH_4)_2SO_4$ 2 g, KH_2PO_4 4 g, Na_2 HPO_4 6 g, $FeSO_4.7H_2O$ 1.0 mg, $CaCl_2$ 1mg, H_3PO_4 10 µg, $MnSO_4$ 10 µg, $ZnSO_4$ 70 µg, $CuSO_4$ 50 µg, MoO_4 10 µg, distilled water 500 ml, pH was adjusted to 7.4.

After autoclaving at 121°C for 15 minutes, the two portions were mixed thoroughly. The *B. cenerea* were cultured into 250 ml Erlenmeyer's conical flasks containing 50 ml broth medium for each and incubated at 28 ºC for 7 days. Medium has the same composition (as above) solidified with agar was dispensed into 9 cm Petri dishes (20 ml per plate); and the Petri dishes were then inoculated with the crude enzyme filtrate in 1 cm diameter wells and incubated for 24 hr at 28°C. After incubation period the dishes were flooded with 1% iodine solution, the excess of iodine was poured off from the plates. Appearance of clear zone around wells indicates the production of pectinase enzyme (Ammar *et al.*, 1995). The activity of enzyme was expressed as (mm), by measuring the clear zone around the wells. Onion plants residues collected, used as cellulosic substrates. The substrate grinded and filterated 20 mesh size. Five grams of onion substrate was subjected for the different treatments such using degrading enzymes produced by *B. cinerea* for 48 h at room temperature or the steam explosion. The treated substrates were utilized for cellulolytic enzyme production of solid state fermentation using *B. cinerea*. Spore number of 1 x 10^6 as inoculation for 5 g material. The composition of media was (g/L): $(NH4)_2SO_4$, 1.4; KH2PO4, 2.0; $CaCl_22H_2O$, 0.3; $MgSO_47H_2O$, 0.3; $FeSO_47H_2O$, 0.005; $MnSO_4H_2O$, 0.0016; $ZnSO_47H_2O$, 0.0014; $CoCl_26H2O$, 0.002; Peptone, 0.1; Tween-80, 0.1. Flasks harvested after 48 h incubation period, Crude enzyme filtrate from the cultured flasks was obtained and centrifuged at 10,000 rpm for 15 min. Crude enzyme extract obtained was analyzed and used for carrying out ethanol production.

Onion residues as substrates for bioethanol production

Onion raw materials were carried out for steam explosion at flask level. One g onion raw material taken in the 100 mL flask, then, the substrate subjected to sudden steam depressurization in an autoclave for 15 psi, 15 min, and 121°C, during that the steam valve was opened to obtain the maximum value of fermentable sugars using least pretreatment time (Sharma *et al.*, 2007). Onion juice for ethanol production was obtained as follows. For each cultivar, onion was pressed in a mechanical juicer and the extract was promptly autoclaved for 30min at 120°C. The extract was then filtered twice, using 6.0 µm pore-size membrane to remove coarse particles and a 0.4 µm pore-size membrane to remove microbes. By this procedure, 60 wt% of the onions processed was recovered as

onion juice. The filtered juice was stored in a refrigerator at 4.0 ºC and used as needed. Then *S. cerevisiae* was cultured in the YPD medium broth in the shaker incubator (VS-8480SF, Korea) at a temperature of 30°C at 120 rpm for 24 h. The growth of yeast was measured by spectrophotometer (600_{nm}) (Horiuchi *et al*., 1999). Then 50 ml of the onion extract and 5 ml of inoculums were added to the four Erlenmeyer flasks at 30°C for 48h. The batch experiments were then carried out at different temperature and pH's. Alcohol fermentation was first performed at 30°C with CO_2 bubbling in order to maintain anaerobic condition (Robati, 2013). Following this, samples for alcohol fermentation were taken and kept at room temperature for 72h. The ethanol production for different onion cultivars by yeast was compared.

Chemical analysis

Reducing sugar estimation: Reducing sugars were analyzed by dinitrosalicylic acid (DNS) method (Miller, 1959). The reducing sugar concentration in the sample was calculated using the standard curve of D-glucose. Ethanol was estimated from samples by dichromate oxidation and thiosulphate titration (Archer *et al.*, 2007). Several mineral elements were determined by inductively coupled plasma atomic emission spectroscopy (ICP, Hitachi, Japan). The amount of sugars was measured by the phenol-sulphuric acid method [8]. Subsequently, the fermented samples were centrifuged (7000 rpm) at 4°C for 6 min and the supernatant were used for the determination of ethanol by HPLC.

RESULTS AND DISCUSSION

Pretreatments like variation in temperature, pH and pretreatment with microorganisms. Steam pretreatments can cause economic effectiveness to remove lignin, and leave the carbohydrate intact (Linde *et al.*, 2007). *S.cerevisiae* is the superior microorganism in ethanol production (Bai *et al.*, 2008). The total sugar contents in the juice of fresh residues of onion cultivars Giza 6, Giza 20, Giza red and Shandaweel were 43.14, 36.0, 41.5 and 32.1 g\l, respectively, that was little similar with previous studies that showed a levels between 46.9 and 79.6 g/l (Horiuchi *et al.*, 1999). The sucrose, fructose and glucose concentrations of Giza 6 residues juice were 16.1, 14.5, and 12.54 g/l, while for Giza 20 were found 14.2, 11.3 and 10.5 g/l, and Giza red were 16.2, 14.3, 11.0 g/l. Moreover, the concentration of these sugars for Shandweel1 11.4, 10.1 and 10.6 g/l, respectively (Table 1).

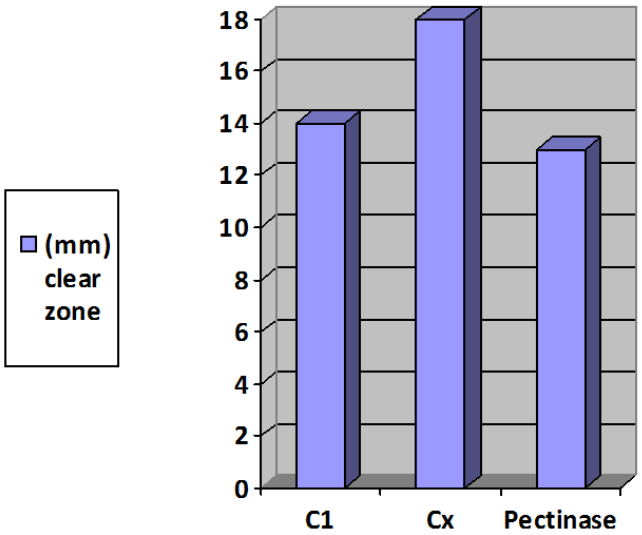
The highest percentage of total sugars was observed for Giza 6, followed by cultivar Giza red. While, the lowest sugars content was founded for Shanweel 1. The cultivar Giza 6 was chosen for ethanol production by *Saccharomyces cerevisiae* at different pH values, temperatures and pretreatments. The maximum production of ethanol, and final ethanol percentage were 6.4 g/L/h, 34 g/L and 86% when onion cultivar Giza 6 was used as a substrate at pH 6.0, and at 30 °C pretreated with *B. cinerea*, many fungi can degrade the cell wall of the plant cells using their ability of the pectin and cellulose hydrolysis (Hancock *et al.*, 1964; Olsson and Hahn-Hägerdal, 1996; El-Said, 2001). The second level of ethanol yield productivity and final ethanol percentage were 6.1 g/L/h, 30 g/L and 82% when onion cultivar Giza 6 was used as a substrate at pH 6.0 and 30 °C pretreated with steam explosion. Accessibility for cellulose components using steam explosion can enhance the effective of fermentation process on the plant cell walls that supported by previous studies (Moniruzzaman, 1996; Sharma *et al.*, 2007; Mtui, 2009).Third ethanol yield and percentage were 5 g/L/h, 25 g/L and 74% at pH 6.0 and 30 °C, the lowest ethanol productivity was obtained for onion cultivar of Giza 6 at pH 5.0 and 35 °C with 3 g/L/h, 15 g/L and 75% (Table 2). That may not typically match with results of previous studies when yeast *S. cerevisiae* and the thermotolerant yeast *S. cerevisiae* were used in the repeated-batch fermentation at 30°C in a molasses medium, ethanol yield in the range of 3.6 to 5 g/L/h, 30.6 g/L and 91.9% were found, respectively (Kida *et al.*, 1992).

Table 1. Chemical analysis of different onion cultivar residues

	Giza 6	Giza 20	Giza red	Shandaweel1
Sucrose	16.1 (g/l)	14.2 (g/l)	16.2 (g/l)	11.4 (g/l)
Fructose	14.5 (g/l)	11.3 (g/l)	14.3 (g/l)	10.1 (g/l)
Glucose	12.54 (g/l)	10.5 (g/l)	11.0 (g/l)	10.6 (g/l)
K	1300 (mg/l)	1100 (mg/l)	1300 (mg/l)	1400 (mg/l)
PO	280 (mg/l)	190 (mg/l)	250 (mg/l)	290 (mg/l)
Mg	56.1 (mg/l)	53.3 (mg/l)	50.1 (mg/l)	55.7 (mg/l)
Ca	67.3 (mg/l)	71.2 (mg/l)	72.3 (mg/l)	59.8 (mg/l)
Na	11.0 (mg/l)	10.0 (mg/l)	13.0 (mg/l)	10.0 (mg/l)
Mn	2.6 (mg/l)	2.3 (mg/l)	3.0 (mg/l)	2.2 (mg/l)
Zn	1.3 (mg/l)	1.1 (mg/l)	1.6 (mg/l)	1.2 (mg/l)
Fe	1.1 (mg/l)	0.9 (mg/l)	1.2 (mg/l)	1.2 (mg/l)

Table 2. Ethanol productivity at different treatments on onion residues of Giza 6 cultivar

Parameters	Giza 6- pH 6		Giza 6- pH 5		Giza 6- pH - 30 °C	Giza 6- pH 6.0 - 30 °C
	30 °C	35 °C	30 °C	35 °C	Steam explosion	Pretreatment *B. cinerea*
Ethanol yield (g/l/h)	5	3.1	4.7	3	6.1	6.4
Final Ethanol yield (g/l)	25	15.2	22.4	15	30.8	34
Ethanol percentage %	74	78	79	75	82	86



*millimeter of clear zone caused by the isolate on the medium

Figure 1. Cellulase and pectinase production of *Botrytis cinerea*

Many minerals were present in onion cultivars Giza 6, Giza 20, Giza red and Shandaweel (Table 1). These results may show a kind of similarity with previous studies on onion plants (Horiuchi *et al.*, 2000). Onion residues found to be rich in nutrients and sugar contents which was a suitable source for biofuel production (Horiuchi *et al.*, 1999).

B. cinerea shown cellulolytic and pectolytic activities on onion plants cell walls (Barkai-Golan *et al*., 1988), the cellulolytic enzymes production was higher than pectolytic by *B. cinerea*. Treatments of onion plants residues gave exhydrolytic and glucosidase activities in this experiments, and the enzymztic activity of pre treatment with *B. cinerea* reflected on the final ethanol productivity (Figure 1).

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