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Fungal Biotreatment of Agro-Industrial Wastes for the Production of Bioethanol in Bioreactor

T.S. El-Tayeb[#], A.A. Abdelhafez, S.H. Ali[*] and E.M. Ramadan[**]
Department of Agricultural Microbiology; [*]Department of Agricultural Chemistry, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima and [**]Biotechnology Laboratory, Heliopolis University, Cairo-Belbies Road, Cairo, Egypt.

BIOETHANOL production from lignocellulosic feedstocks is considered a promising strategy to increase global production of biofuels without impacting food supplies. This work aimed to evaluate bioethanol production by baker's yeast using a medium containing the hydrolysate of fungal biotreatment of five different lignocellulosic feedstocks with some amendments. The pretreatment of lignocellulosic feedstocks using 5 % w/v NaOH, 1 % v/v H_2SO_4 and sodium hypochlorite: H_2O_2 (10:1) prior to fungal biotreatment was studied. For bioethanol production, batch, fed-batch (two strategies) and continuous cultivations of baker's yeast on the fungal biotreated rice straw hydrolysate was evaluated in bioreactor. In batch and pulsed fed-batch cultivations, the highest bioethanol concentration, conversion coefficient, bioethanol yield and productivity were (0.41 % v/v, 36.9 % v/w, 36.9 % v/w and 0.114 ml/l/h, respectively), while in fed-batch cultivation with continuous feeding these parameters were (0.45 % v/v, 40 % v/w, 40.5 v/w % and 0.015 ml/l/h, respectively). The highest bioethanol concentration (0.52 % v/v) was obtained in continuous culture at dilution rate of 0.03 h^{-1}, while conversion coefficient, yield and productivity were 31.2 % v/w, 31.4 % v/w and 0.022 ml/l/h, respectively.

Keywords: Bioethanol, Agro-industrial wastes, Biotreatment, Baker's yeast, *Trichoderma viride* EMCC 107.

Lignocellulosic complex is the most abundant biopolymer on Earth. Many lignocellulosic feedstocks have been tested for bioethanol production. Processing of lignocellulosics is an essential step in releasing fermentable carbohydrate components for the production of bioethanol. This goal can be accomplished by combining pretreatment and hydrolysis steps that involve physical, chemical, thermal and/or enzymatic treatments. The main effect of pretreatment of lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan, thus increasing the porosity of biomass. Several research approaches are being carried out to increase bioethanol yields from

[#]**Corresponding author mailing address:** Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, 11241, Cairo, Egypt. E-mail: tarekeltayeb@yahoo.com.

available feedstocks. In this respect, the alkali treatment of rice straw with NaOH, peracetic acid (PA), and sodium chlorite ($NaC1O_2$) resulted in a remarkable decrease in hemicellulose as well as lignin. The treated straw was biotreated with the culture filtrate of *Trichoderma reesei.* The degree of enzymatic hydrolyzation relative to the amount of residual straw was 69 % (treated with 0.2 N NaOH), 42 % (treated with 20 % PA), and 50 % (treated with $NaC1O_2$) (Taniguchi *et al.,* 2005). Jakobsson (2005) pretreated wheat straw with steam at different temperatures (190, 200, 210ºC) and residence times (2, 5, 10 min) to make cellulose more accessible to enzymes. The straw was impregnated with sulphuric acid before pretreatment. The pretreatment at 190ºC for 10 min resulted in the best overall yield of fermentable sugars. In another study, rice straw was pretreated using a solution containing 20 ml sodium hypochlorite and 100 ml hydrogen peroxide for 1 g rice straw resulted in 47.3 mg glucose and 72.5 mg xylose. The latter treatment was an essential step for efficient bioethanol production with *Saccharomyces cerevisiae* and *Pichia stipitis.* With initial 5 % sugars concentration, the final ethanol concentration was about 1.67 % (Choi *et al*., 2009). Silva *et al.* (2013) pretreated rice straw in alkaline medium (pH 8) in the presence of H_2O_2, increasing yeast fermentability of rice straw hydrolysate to double the bioethanol volumetric productivity in relation to the untreated hydrolysate.

Fungal biotreatment of lignocellulosic feedstocks for the obtainment of fermentable sugars for bioethanol production was investigated by many authors; Zhang *et al.* (2007) observed a maximum saccharification yield of 37 % (w/w) from biotreated bamboo residues with *Coriolus versicolor*. Biotreatment of wheat straw for 60 d with *P. ostreatus* resulted in 33 % conversion of cellulose to glucose (Taniguchi *et al.,* 1982). Manjunath & Geeta (2007) biotreated sugarcane bagasse, paddy straw and wheat straw with the fungi *Phanerochaete chrysosporium* and *Pleurotus* spp. The filtrate fermentation of these materials showed maximum production of bioethanol on baggase (0.08 % w/w) by *Zymomonas mobilis*. Wan & Li (2010) was able to convert 57.7 % of corn stover to glucose after biotreatment with the fungus *Ceriporiopsis subvermispora* for 18 d at 28°C and the highest overall bioethanol yield was 57.80 %. In another investigation, bioethanol was produced from hydrolysates of grass, hemp stem, wheat straw, newspaper, and cellulose resulted from pretreatment with acid or alkali and the enzymes Celluclast and Novozymes 188. Chemical pretreatment increased bioethanol yields from 0.8 mM/g to 3.3 mM/g from alkali-pretreated straw (Jessen & Orlygsson, 2012).

Fermentation process development has great impact on efficient bioethanol production (Bai *et al.,* 2004 and Laopaiboon *et al.,* 2007). Typically, bioethanol production is often conducted in batch mode. However, the batch process has many disadvantages, particularly when the microorganisms are either slow growing or strongly affected by product inhibition. A report studied batch bioethanol production by *Sacch. cerevisiae* in bioreactor from raw sugar beet juice and the bioethanol yield was 59.89 g/l and production efficiency 78.8 %,

and in fed-batch process the yield was 92.78 g/l and efficiency 93.4 %. At the same time, batch bioethanol production from raw sugar beet cossettes resulted in the highest bioethanol yield of 54.53 g/l and production efficiency of 79.5 % (Pavlečić *et al.*, 2010).

Control of the flow rate of medium feed is quite advantageous because the inhibitory effect caused by high substrate or product concentrations in the fermentation broth can be minimized. Souza *et al.* (2007a) proposed a pulse fed-batch strategy for bioethanol production and observed greater bioethanol productivity and lesser biomass formation during fermentation. Souza *et al.* (2007b) improved bioethanol production in a pulse fed-batch cultures. They resolved the lethal effect of bioethanol on cell survival by reducing the sucrose concentration in the fermentation system. Continuous processes permit many advantages including the decrease of product inhibition effect. Purwadi *et al.* (2007) employed a continuous cultivation system using a strain of *Sacch. cerevisiae* to ferment a non-detoxified spruce hydrolysate. At dilution rate up to 0.52 h^{-1}, a bioethanol yield of 0.42 – 0.46 g/g consumed sugars was achieved, and the residual sugar concentration was less than 6 % of the initial fermentable sugars. Saha & Cotta (2005) investigated the continuous production of bioethanol from alkaline peroxide pretreated and enzymatically saccharified wheat straw under various conditions. The average bioethanol produced from the available sugars (21.9 – 47.8 g/l) ranged from 8.8 to 17.3 g/l (0.28 – 0.45 g/g available sugars, 0.31 – 0.48 g/g sugar consumed) with bioethanol productivity of 0.27 – 0.78 g/l/h in a set of 14 continuous culture runs (16 – 105 days).

The aim of this work is to evaluate pretreatment and fungal biotreatment of some agro-industrial wastes in either shake flasks or bioreactor in order to improve the efficiency of bioethanol fermentation by different strategies. Bioethanol production in bioreactor was carried out, using baker's yeast, in batch, fed-batch and continuous cultivation techniques.

Materials and Methods

Agro-industrial wastes

Rice straw and corn stalks were collected from farms of rice and corn in El–Beheira Governorate (located in Lower Egypt in the Delta of the Nile north of Cairo. Sugar beet waste and sugarcane bagasse were obtained from the Sugars Refinery Factory at El–Beheira Governorate and sugarcane fresh syrup shops in Cairo, respectively. Sawdust was gathered from the local joinery atelier at Heliopolis University, Cairo, Egypt. Corn stalks were coarsely crushed using a laboratory hammer mill (Retsch GmbH & Co. KG, Germany). The other agro-industrial wastes were chopped into small pieces using a shredder and then ground to pass through 1.5 mm screens. All samples were homogenized and oven-dried at 45°C. The dried materials were stored in air tight containers at room temperature before use.

Microorganisms

Cellulose degrading fungi: *Trichoderma viride* EMCC 107 were obtained from Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Bioethanol producing organism: Fresh commercial baker's yeast was purchased from a local bread bakery in Cairo.

Pretreatment of agro-industrial wastes before fungal biotreatment

Five grams of each residue (rice straw, corn stalks, sawdust, sugar beet waste or sugarcane bagasse) was placed in 250 ml conical flasks. Each flask contained 100 ml of either of the following solutions: 5 % w/v NaOH, 1 % v/v H_2SO_4 and sodium hypochlorite: H_2O_2 (10:1). Treatments with 5 % w/v NaOH, 1 % v/v H_2SO_4 were incubated for 2 h, while the treatment with sodium hypochlorite: H_2O_2 (10:1) was incubated for 24 h. The flasks were placed on a shaker at 30°C with gentle mixing (120 rpm) for 1 h. After treatment, all residues were washed carefully with tap water and rinsed adequately and the final pH was adjusted to 7.0 before using in biotreatment experiments. The most efficient pretreatment process was conducted on sufficient amount of rice straw needed for biotreatment in bioreactor.

Biotreatment of agro-industrial wastes

T. viride EMCC 107 was grown and maintained on potato dextrose agar (PDA) slants. Fungal cultures were inoculated onto PDA medium in Petri plates. After 4–5 days of incubation at 28°C, cultures were used for inoculation of medium containing different pretreated agro-industrial wastes. The fungal biotreatment of agro-industrial wastes in shake flasks prior to bioethanol production was carried out in submerged culture according to the method described in El-Tayeb *et al.* (2012). Shake flasks tests were performed in triplicates. For fungal biotreatment of rice straw in bioreactor, a 10 liter dished bottom bioreactor MS-1 (Major Science Instruments) was used. The vessel of bioreactor was equipped with lipseal stirrer assembly, automatic pH controller, automatic dissolved O_2 and automatic temperature controller. Bioreactor containing 5 % w/v pretreated rice straw was filled with medium described by El-Tayeb *et al.* (2012) and sterilized at 121°C for 25 min. After inoculation, the final working volume was adjusted to 7 liters. For inoculum preparation, five discs (5 mm diam.) of the plate cultures of *T. viride* EMCC 107 were inoculated into 700 ml of 2.0 % (w/v) malt extract medium in 1000 ml conical flasks and incubated in shaker incubator at 28°C and 150 rpm for 5 d. The liquid inoculum was introduced aseptically into bioreactor. Fungal growth and rice straw degradation were monitored during 15 d. The temperature and pH were kept constant at 28°C and 7.0, respectively, with aeration at 1 vvm and agitation at 200 rpm. After biotreatment, the residual materials were separated by filtration through filter cloth then through Whatman filter paper No.1. The filtrates were used for total fermentable sugars determination (AOAC, 2007) and bioethanol production.

Bioethanol production from biotreated agro-industrial wastes

Bioethanol production was conducted in 250 ml conical flasks containing 100 ml of filtered hydrolysate resulting from the fungal biotreatment of different lignocellulosic feedstocks as basal medium. Flasks were amended with the following components (g/100 ml): yeast extract 1, $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.1, $MgCl_2$ 0.1, and pH was adjusted to 5.0 (El-Tayeb *et al.,* 2012). Baker's yeast was inoculated at 2 % w/v in either flasks or bioreactor cultivations. Flasks were incubated in an anaerobic incubator (Hirayama Manufacturing Corp., Tokyo, Japan) at 30°C for 4 d. The overall bioethanol concentration was determined colormetrically according to the method of Lau & Luk (1994).

Effect of medium composition on bioethanol formation

For the optimization of bioethanol production medium, 250 ml conical flasks containing 100 ml of filtered hydrolysate derived from the fungal biotreatment of rice straw were used as basal medium. To the basal medium, six different amendments were added to flasks (including control) according to the following formulas: (I)YE 0.0 + $(NH_4)_2SO_4$ 0.5 + KH_2PO_4 1.0 + $MgCl_2$ 0.1, (II) YE 0.5 + $(NH_4)_2SO_4$ 0.5 + KH_2PO_4 1.0 + $MgCl_2$ 0.1, (III)YE 0.5 + $(NH_4)_2SO_4$ 0.1 + KH_2PO_4 1.0 + $MgCl_2$ 0.1, (IV)YE 1.0 + $(NH_4)_2SO_4$ 0.5 + KH_2PO_4 1.0 + $MgCl_2$ 0.1, (V)YE 1.0 + $(NH_4)_2SO_4$ 0.1 + KH_2PO_4 1.0 + $MgCl_2$ 0.1 and (VI)YE 1.0 + $(NH_4)_2SO_4$ 0.1 + KH_2PO_4 0.1 + $MgCl_2$ 0.1 (control). Conditions of bioethanol production were carried out as previously mentioned. The medium formula that achieved the highest bioethanol concentration was used in bioreactor cultivations with a final working volume of 5 liters.

Bioreactor conditions

The bioreactor described above was used for bioethanol production in batch, pulsed fed-batch, continuous feeding and continuous cultivations with additional assembly of a multi–channel peristaltic pump (for feeding) and all the accessories for continuous cultivation. In all cases, the final working volume was 5 liters. In batch culture, the medium (5 liters) of filtered hydrolysate, derived from the fungal biotreatment of rice straw according to the conditions previously mentioned, was added totally to bioreactor before sterilization, while in pulsed fed-batch cultivation the medium (5 liters) was divided to equal amounts and added to fermentation vessel at 3 h intervals during 33 h of cultivation period. In continuous feeding, the medium was fed continuously during the first 33 h of cultivation at a constant addition rate of 151.5 ml/h. The temperature was controlled at 30°C, whereas pH was controlled at 5.5 with 2 N NaOH during fermentation. Samples (10 ml) were taken from the growing culture periodically under aseptic conditions to determine bioethanol and sugars concentrations.

In continuous culture, the cultivation was carried out as batch culture for 48 h at 30°C then fresh medium was pumped to growing culture at different flow rates of 50, 100, 150 and 200 ml/h to give 0.01, 0.02, 0.03 and 0.04 h^{-1} dilution rates, respectively. Appropriate culture volumes were withdrawn from the fermentation vessel at a given time to keep the total volume of culture constant at five liters.

Each steady state was kept running for at least four days intervals. Three samples were taken aseptically at each steady state to determine bioethanol concentration and average response was calculated.

Statistical analysis

Duncan`s Multiple Range Test was used to test significance of means according to IBM® SPSS® statistics software (IBM, 2011).

Results and Discussion

It is evident the importance of lignocellulosic biomass as a feedstock for bioethanol production. The main processing challenge in producing bioethanol from lignocellulosic biomass is the feedstock pretreatment. In our previous work (El-Tayeb *et al.,* 2012), it was observed that fungal biotreatment of agro-industrial wastes may be a useful tool for lowering the production costs of bioethanol from lignocellulosic feedstocks comparing with acid hydrolysis treatments. Besides that, the biotreatment of different feedstocks led to a decrease in the final bioethanol concentration comparing with acid hydrolysis treatments (El-Tayeb *et al.,* 2012). Attempts to increase the final bioethanol concentration on fungal biotreated lignocellulosic feedstocks were examined throughout this investigation.

Pretreatment of agro-industrial wastes before fungal biotreatment

In the present study, pretreatment with 5 % w/v NaOH, 1 % v/v H_2SO_4 and with sodium hypochlorite: H_2O_2 (10:1) were compared separately on different agro-industrial wastes before the fungal biotreatment by *Trichoderma viride* EMCC 107. In general, the pretreatment with sodium hypochlorite: H_2O_2 (10:1) significantly increased the conversion percentage of agro-industrial wastes to total sugars, compared to control (without pretreatment), which reflected on enhancing the bioethanol production process. This pretreatment achieved the highest bioethanol concentration on all agro-industrial wastes comparing with control (Table 1). The highest bioethanol concentration (0.55 % v/v) was obtained on sugar beet waste (5 % w/v) pretreated with sodium hypochlorite: H_2O_2 (10:1), followed by sugarcane baggase (5 % w/v) influenced with the same pretreatment (0.52 % v/v). These records increased bioethanol concentration by 25 % and 26.8 %, respectively comparing with control. The effect of the other pretreatments varied greatly. The pretreatment with 5 % NaOH or 1 % v/v H_2SO_4 decreased significantly the final bioethanol concentration in most cases. Accordingly, pretreatment of rice straw with sodium hypochlorite: H_2O_2 (10:1) was conducted throughout the rest of this investigation.

Many authors have reported various kinds of pretreatments of different agro-industrial wastes prior to hydrolysis process for obtainment of fermentable sugars for bioethanol production. Shrestha *et al.* (2009) pretreated corn fiber with 2 % NaOH (w/w) at 30°C for 2 h. This pretreatment resulted in higher glucose yields following fungal saccharification of corn fiber. Bioethanol yields were 2.6 g, 2.9 g

and 5.5 g bioethanol / 100 g of corn fiber, from *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* and *T. reesei*, respectively. Kang *et al.* (2011) pretreated rice straw using a solution containing 0.6 % hypochlorite and 25 % hydrogen peroxide to obtain 406.8 mg D-glucose and 224.0 mg D-xylose from 1 g of rice straw. The fermentation of enzymatic hydrolysates containing 8.14 g/l D-glucose and 4.49 g/l d-xylose with *Pichia stipitis* generated 3.65 g/l of bioethanol with a corresponding yield of 0.37 g/g. The maximum possible bioethanol conversion rate was 72.54 %. Lin & Lee (2011) pretreated the sticks of rice straw with 10 % NaOH at room temperature prior to enzymatic hydrolysis and bioethanol production. Bioethanol production on alkali pretreated rice straw at 40°C produced a bioethanol concentration of 29 g/l and a bioethanol yield of 86 % (based on glucose content in the pretreated raw material) in a 72 h reaction.

TABLE 1. Effect of pretreatment of agro-industrial wastes before fungal biotreatment on their conversion percentage to total sugars and the equivalent bioethanol production by baker's yeast using shake flasks technique.

Agro-industrial wastes (5 % w/v)	Pretreatment of agro-industrial wastes before fungal biotreatment	Conversion percentage of agro-industrial wastes to total sugars (% w/w)*	Bioethanol concentration (% v/v)
Rice straw	5 % w/v NaOH	10.8^{n}	0.08^{fg}
	1 % v/v H_2SO_4	12.5^{l}	0.1^{f}
	NaClO : H_2O_2 (10:1)	17.3^{h}	0.3^{c}
	Control	12.1^{l}	0.2^{e}
Corn stalks	5 % w/v NaOH	9.2^{o}	0.05^{h}
	1 % v/v H_2SO_4	12.2^{l}	0.09^{f}
	NaClO : H_2O_2 (10:1)	19.1^{g}	0.32^{c}
	Control	13.2^{j}	0.1^{f}
Sawdust	5 % w/v NaOH	10.4^{n}	0.05^{h}
	1 % v/v H_2SO_4	11.7^{m}	0.08^{g}
	NaClO : H_2O_2 (10:1)	13.1^{k}	0.1^{f}
	Control	9.5^{o}	0.05^{h}
Sugar beet waste	5 % w/v NaOH	30.4^{d}	0.22^{e}
	1 % v/v H_2SO_4	31.3^{c}	0.25^{d}
	NaClO : H_2O_2 (10:1)	39.5^{a}	0.55^{a}
	Control	21.2^{f}	0.44^{b}
Sugarcane bagasse	5 % w/v NaOH	15^{i}	0.18^{ef}
	1 % v/v H_2SO_4	32.1^{b}	0.23^{e}
	NaClO : H_2O_2 (10:1)	30.3^{d}	0.52^{a}
	Control	22.4^{e}	0.41^{c}

*(% w/w) = percentage based on dry weight.
Control = agro-industrial wastes without pretreatment.
The values are mean of three replicates. Standard deviation was within 10 %.
Values in the same column followed by the same letter(s) do not significantly differ from each other according to Duncan's at 5 % level.

Optimization of medium composition for bioethanol formation

A great number of microorganisms are capable of bioethanol formation on different productive media among which *Sacch. cerevisiae* (baker's yeast) is the most frequently and traditionally used organism (Barcelos *et al.*, 2011). For the optimization of medium used for bioethanol production by baker's yeast, five different modifications were tested by changing the concentrations of medium components added to the filtered fungal hydrolysate obtained previously by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw (Table 2). The highest final bioethanol concentration was obtained in a medium containing the following components (g/100 ml): yeast extract 1.0, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 1.0 and $MgCl_2$ 0.1. This modification increased bioethanol concentration (0.36 % v/v) by 24.1 % comparing with control and was applied for the further experiments. Most other combinations of productive medium nutrients decreased significantly final bioethanol concentration comparing with control. Several authors used many bioethanol producing organisms on different productive media. Rajoka *et al.* (2005) reported bioethanol productivity (7.2 g/l/h), product yield (0.44 g bioethanol/g substrate utilized) and specific bioethanol yield (19.0 g bioethanol/g cells) in a medium containing molasses (15 % reducing sugars) and the following nutrients (g/l): $(NH_4)_2SO_4$, 2.5; $MgSO_4$, 1.0 and KH_2PO_4, 2.0 by *Sacch. cerevisiae* ATCC 26602 in a completely controlled bioreactor. In another study, batch bioethanol fermentation was carried out on sweet sorghum juice by *Sacch. cerevisiae* NP 01 in a 500 ml air-locked conical flask. The maximum bioethanol production efficiency was obtained when 9 g/l of yeast extract was supplemented to the juice. The bioethanol concentration, productivity and yield were 120.24 g/l, 3.01 g/l/h and 0.49, respectively (Nuanpeng *et al.*, 2011). Landaeta *et al.* (2013) used a base medium contained (g/l): glucose 25, yeast extract 1, KH_2PO_4 1, $(NH_4)_2SO_4$ 0.4 and $MgSO_47H_2O$ 0.08 for bioethanol production (0.48 g/l/h) by *Sacch. cerevisiae* (NRRL Y-265). Maruthai *et al.* (2012) studied the effect of medium components (a total of fifteen components) on bioethanol production from waste cashew apple juice using yeast *Sacch. diasticus*. In general, initial substrate concentration significantly influenced the microbial growth and product formation. Of the medium components evaluated, yeast extract, $(NH_4)_2SO_4$, and malt extract showed significant effect on ethanol fermentation. Maximum bioethanol (15.3 g/l) was obtained at the optimum medium composition.

Bioethanol production in bioreactor

Rice straw is an attractive lignocellulosic material for bioethanol production since it is one of the most abundant renewable resources. It has several characteristics, such as high cellulose and hemicelluloses content that can be readily hydrolysed into fermentable sugars. According to the data obtained in the current work, rice straw was chosen as the most considerable cellulosic material for bioethanol production. The two stages of bioethanol production, which consisted of feedstock degradation and bioethanol fermentation, were conducted on rice straw to further optimization in batch, fed-batch (two strategies) and continuous cultures in bioreactor.

TABLE 2. Enhancing bioethanol production by optimizing the composition of production medium containing rice straw hydrolysate* and inoculated with baker's yeast.

Nutrients supplemented to filtered rice straw hydrolysate (bioethanol production medium) (g/100 ml)	Bioethanol concentration (% v/v)
YE 0.0 + $(NH_4)_2SO_4$ 0.5 + KH_2PO_4 1.0 + $MgCl_2$ 0.1	0.2^{f}
YE 0.5 + $(NH_4)_2SO_4$ 0.5 + KH_2PO_4 1.0 + $MgCl_2$ 0.1	0.25^{e}
YE 0.5 + $(NH_4)_2SO_4$ 0.1 + KH_2PO_4 1.0 + $MgCl_2$ 0.1	0.28^{d}
YE 1.0 + $(NH_4)_2SO_4$ 0.5 + KH_2PO_4 1.0 + $MgCl_2$ 0.1	0.36^{a}
YE 1.0 + $(NH_4)_2SO_4$ 0.1 + KH_2PO_4 1.0 + $MgCl_2$ 0.1	0.31^{b}
YE 1.0 + $(NH_4)_2SO_4$ 0.1 + KH_2PO_4 0.1 + $MgCl_2$ 0.1 (control)	0.29^{c}

*Filtered hydrolysate = obtained by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw in conical flasks.
YE = Yeast extract.
Control = original bioethanol production medium.
The values are mean of three replicates. Standard deviation was within 10 %.
Values in the same column followed by the same letter(s) do not significantly differ from each other according to Duncan's at 5 % level.

Batch production

Biodegradation of rice straw using the fungus *T. viride* EMCC 107 was conducted in submerged batch culture. The growth of the fungus increased greatly in bioreactor comparing with that obtained in conical flasks propagation. At the end of culturing period, the final conversion percentage of rice straw to total sugars was 22.2 % w/w, which was higher than that obtained in conical flasks experiments (17.3 % w/w, Table 1). Hydrolysate containing fermentable sugars was obtained by filtration of fungal culture and used for bioethanol production by baker's yeast in bioreactor. At the end of fermentation period, bioethanol concentration was 0.41 % v/v, which represented an increase by 13.89 % comparing with that obtained in batch production in conical flasks (0.36 % v/v, Table 2). This result indicated that rice straw degradation in bioreactor by *T. viride* EMCC 107 was higher than that obtained in conical flasks which also consequently reflected on increasing the final bioethanol concentration. The highest figures of conversion coefficient, bioethanol yield and productivity at the end of fermentation period, were (36.9 % v/w, 36.9 % v/w and 0.114 ml/l/h, respectively). In a related work, Nuanpeng *et al.* (2011) scaled up batch bioethanol fermentation in a 5 liter bioreactor at an agitation rate of 100 rev min^{-1}. They revealed that bioethanol concentration, productivity and yield were 139.51 g/l, 3.49 g/l/h and 0.49, respectively. In another report, *Sacch. cerevisiae* was used for the production of bioethanol from sorghum grains hydrolysate (250 g/l glucose) in batch bioreactor. The maximum bioethanol concentration produced was 106 g/l in 24 h, resulting in a productivity of 4.4 g/l/h and a product yield based on the substrate consumed (0.499 g/g) (Barcelos *et al.*, 2011).

Pulsed fed-batch and continuous feeding

In fermentation processes, cell growth and/or product formation may be inhibited by substrate inhibitory materials concentrations, thus substrate is intermittently fed to the culture system in order to maintain the substrate concentration below a certain level for enhancement of biological and metabolic activity. Empirical feeding techniques of the filtered fungal hydrolysate of rice straw have been developed to improve the bioethanol production process. The optimization of feeding policy plays a crucial role for increasing both productivity and bioethanol yield of fed-batch fermentations. This issue was analyzed in a previous review (Cardona & Sànchez, 2007). In present investigation, two feeding techniques, including pulsed and continuous feeding were conducted to determine the most efficient technique for high bioethanol production by baker's yeast. Pulsed addition of the filtered hydrolysate was carried out every three hours during two days of bioethanol production period (Table 3). Bioethanol concentration and consumed sugars increased gradually during fermentation period. At the end of fermentation period (36 – 48 h), the highest bioethanol concentration and bioethanol yield were obtained (0.41 % v/v and 36.9 % v/w, respectively), whereas, the highest productivity was recorded after 33 h (0.0115 ml/l/h). The maximum conversion coefficient (39.7 % v/w) was obtained after 30 h. Data in Table 4 show bioethanol production by baker's yeast grown on rice straw hydrolysate by continuous fed-batch culture at rate of 151.5 ml/h during 2 days of fermentation. The maximum bioethanol concentration was obtained after 30 hr of incubation (0.45 % v/v). The corresponding figures of conversion coefficient, bioethanol yield and productivity were 40.5 % v/w, 40.5 v/w % and 0.015 ml/l/h, respectively. Bioethanol concentration was constant at 0.45 % v/v throughout the last 18 h of fermentation period, while bioethanol productivity was decreasing throughout this period. Generally, it could be concluded that the continuous feeding at 151.5 ml/h during 2 days, was favorable for high bioethanol production than that recorded in pulsed feeding technique. In spite of the low concentration of sugars in the feeding liquor which sometimes discourages the strategy of fed-batch cultivation in general, this strategy increased the final bioethanol concentration which might be due to lowering of some inhibitory materials in liquor. In a previous report, bioethanol fermentation of sunflower seed hull hydrolysate was carried out in a batch bioreactor system using *Pichia stipitis* NRRLY-7124. The highest bioethanol accumulation, 9.66 g/l, and a yield of 0.41 g/g were achieved at the lowest tested flow rate, 2.28 /vv/min, from 35 g/l reducing sugars (Okur & Saraçoğlu, 2006). Kumoro *et al.* (2009) investigated the effect of different glucose feeding rates on bioethanol fermentation in fed-batch culture. The 2 g/l/h glucose concentration feeding rate gave higher bioethanol yield (2.47 g bioethanol / g glucose), with respect to substrate consumed, as compared to 8 g/l/h (0.23 g bioethanol / g glucose). In batch culture, bioethanol yield was 0.81 g/g glucose. The specific glucose consumption rate and specific bioethanol production rate for the fed-batch fermentation, at 2 g/l/h glucose feeding rate, were 1.20 h^{-1} and 0.0009 h^{-1}, respectively.

TABLE 3. Time course of bioethanol production by baker's yeast in a pulsed fed-batch culture with filtered fungal hydrolysate obtained by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw in bioreactor.

Time (h)	Added solution (ml)	Total added sugars (g)	Bioethanol concentration (% v/v)	Consumed sugars (% w/v)	Residual sugars (% w/v)	Conversion coefficient (% v/w)	Bioethanol yield (% v/w)	Productivity (ml/l/h)
0	625	6.93	0.0	0.0	1.11	-	-	-
3	625	13.87	0.011	0.05	1.06	22	0.9	0.0037
6	625	20.79	0.042	0.14	0.96	30	3.7	0.007
9	625	27.72	0.08	0.21	0.91	38	7.2	0.0089
24	625	34.65	0.15	0.42	0.68	35.7	13.5	0.0063
27	625	41.58	0.21	0.57	0.53	36.8	18.9	0.0078
30	625	48.51	0.29	0.73	0.36	39.7	26.1	0.0097
33	625	55.44	0.38	1.08	0.01	35.2	34.2	0.0115
36	0.0	55.44	0.41	1.11	0.0	36.9	36.9	0.0114
48	0.0	55.44	0.41	1.11	0.0	36.9	36.9	0.0085

The values are mean of three samples determinations. Standard deviation was within 5 %.
Added sugars concentration at each time interval = 1.11 % w/v.
Conversion coefficient (%) = amount of bioethanol produced (% w/v) x 100 / sugars consumed (% w/v) (Gamal *et al.*, 2013).
Bioethanol yield (%) = amount of bioethanol produced (% v/v) x 100 / initial sugars concentration (% w/v) (Gamal *et al.*, 2013).
Productivity (P) = amount of bioethanol produced (% v/v) / fermentation time (h) = ml/l/h (Gamal *et al.*, 2013).

TABLE 4. Time course of bioethanol production by baker's yeast in a continuous fed-batch culture (specific addition rate at 151.5 ml/h) with filtered fungal hydrolysate obtained by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw in bioreactor.

Time (h)	Added solution (ml)	Total added sugars (g)	Bioethanol concentration (% v/v)	Consumed sugars (% w/v)	Residual sugars (% w/v)	Conversion coefficient (% v/w)	Bioethanol yield (% v/w)	Productivity (ml/l/h)
0	0.0	0.0	0.0	0.0	1.11	-	-	-
3	714.3	7.93	0.04	0.11	0.99	36.4	3.6	0.013
6	1428.6	15.8	0.12	0.28	0.81	42.9	10.8	0.02
9	2142.9	23.8	0.21	0.55	0.55	38.2	18.9	0.023
24	2857.2	31.7	0.31	0.75	0.34	41.3	27.9	0.013
27	3571.5	39.6	0.39	0.95	0.13	41.0	35.1	0.014
30	4285.8	47.6	0.45	1.11	0.0	40.5	40.5	0.015
33	5000	55.5	0.45	1.11	0.0	40.5	40.5	0.014
36	0.0	0.0	0.45	1.11	0.0	40.5	40.5	0.013
48	0.0	0.0	0.45	1.11	0.0	40.5	40.5	0.009

The values are mean of three samples determinations. Standard deviation was within 5 %.
Added sugars concentration at each time interval= 1.11 % w/v.
Conversion coefficient (%) = amount of bioethanol produced (% v/v) x 100 / sugars consumed (% w/v) (Gamal *et al.*, 2013).
Bioethanol yield (%) = amount of bioethanol produced (% v/v) x 100 / initial sugars concentration (% w/v) (Gamal *et al.*, 2013).
Productivity (P) = amount of bioethanol produced (% v/v) / fermentation time (h) = ml/l/h (Gamal *et al.*, 2013).

Continuous culture

The design and development of continuous fermentation systems have allowed the implementation of more cost effective processes. Continuous processes have several advantages compared to conventional batch processes mainly due to the reduced construction costs of the bioreactors, lower maintenance and operation requirements, better process control, and higher productivities.

Bioethanol production by baker's yeast was studied in continuous culture at different dilution rates (steady states 0.01, 0.02, 0.03 and 0.04 h^{-1}) in bioreactor. Bioethanol production and productivity were expressed as a function of dilution rates. The filtered rice straw hydrolysate was added to the bioreactor at different flow rates ranged from 50 to 200 ml/h, after 48 h of incubation. Results in Tables 5, 6, 7 and 8 show that variation in dilution rate, resulted in changes in the steady state of conversion coefficient, bioethanol yield and productivity. Bioethanol concentration increased with increasing dilution rate from 0.01 h^{-1} to 0.03 h^{-1} and remained constant for four days, while washing out was observed at 0.04 h^{-1}. Bioethanol concentration outlet ranged from 0.155 to 0.185 ml/h at dilution rate 0.01 h^{-1}, whereas they ranged from 0.44 to 0.48 ml/h at dilution rate 0.02 h^{-1}. The mean value of the highest amount of bioethanol concentration outlet (0.765 ml/h) was attained at 0.03 h^{-1} dilution rate. At 0.04 h^{-1} dilution rate, where no steady state was observed, bioethanol concentration outlet was decreased from 0.38 to 0.02 ml/h, during four days of incubation period. Sugars input were 0.555, 1.11, 1.665, and 2.22 g/h for 0.01, 0.02, 0.03 and 0.04 h^{-1} dilution rates, respectively. Consumed sugars (g/h) at different dilution rates were increased with the increase of sugars input till 0.03 h^{-1}. Conversion coefficient, bioethanol yield and bioethanol productivity were increased with increasing of dilution rate (sugars input), reaching the maximum at 0.03 h^{-1} (30.6 % v/w, 30.8 % v/w and 0.021 ml/l/h, respectively), then decreased at 0.04 h^{-1} dilution rate (where no steady state was observed). Accordingly, it could be stated that the maximum dilution rate to be used, is 0.03 h^{-1} for giving maximum bioethanol productivity.

TABLE 5. Bioethanol production by baker's yeast in continuous culture at 0.01 h^{-1} dilution rate (50 ml filtered hydrolysate* / h flow rate / 5000 ml culture).

Time (days)	Sugars in put (g/h)	Sugars out let (g/h)	Consumed sugars (g/h)	Bioethanol concentration out let (% v/v)	Bioethanol concentration out let (ml/h)	Conversion coefficient (% v/w)	Bioethanol yield (% v/w)	Productivity (ml/l/h)
1	0.555	0.002	0.553	0.37	0.185	66.9	66.67	0.015
2	0.555	0.001	0.552	0.35	0.175	63.4	63.06	0.014
3	0.555	0.002	0.553	0.32	0.16	57.8	57.66	0.013
4	0.555	0.001	0.554	0.31	0.155	55.9	55.86	0.013
Means	0.555	0.0015	0.553	0.3375	0.169	61	60.81	0.01375

*Filtered hydrolysate = obtained by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw in bioreactor.
The values are mean of three samples determinations. Standard deviation was within 5 %.
Conversion coefficient (%) = amount of bioethanol produced (% v/v) x 100 / sugars consumed (% w/v) (Gamal *et al.*, 2013).
Bioethanol yield (%) = amount of bioethanol produced (% v/v) x 100 / initial sugars concentration (% w/v) (Gamal *et al.*, 2013).
Productivity (P) = amount of bioethanol produced (% v/v) / fermentation time (h) = ml/l/h (Gamal *et al.*, 2013).

TABLE 6. Bioethanol production by baker's yeast in continuous culture at 0.02 h^{-1} dilution rate (100 ml filtered hydrolysate* / h flow rate / 5000 ml culture).

Time (days)	Sugars in put (g/h)	Sugars out let (g/h)	Consumed sugars (g/h)	Bioethanol concentration out let (% v/v)	Bioethanol concentration out let (ml/h)	Conversion coefficient (% v/w)	Bioethanol yield (% v/w)	Productivity (ml/l/h)
1	1.11	0.004	1.106	0.48	0.48	43.2	43.2	0.02
2	1.11	0.005	1.105	0.46	0.46	41.4	41.4	0.019
3	1.11	0.004	1.106	0.44	0.44	39.6	39.6	0.018
4	1.11	0.006	1.106	0.46	0.46	41.4	41.4	0.019
Means	1.11	0.0048	1.1058	0.46	0.46	41.4	41.4	0.019

*Filtered hydrolysate = obtained by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw in bioreactor.
The values are mean of three samples determinations. Standard deviation was within 5 %.
Conversion coefficient (%) = amount of bioethanol produced (% v/v) x 100 / sugars consumed (% w/v) (Gamal *et al.*, 2013).
Bioethanol yield (%) = amount of bioethanol produced (% v/v) x 100 / initial sugars concentration (% w/v) (Gamal *et al.*, 2013).
Productivity (P) = amount of bioethanol produced (% v/v) / fermentation time (h) = ml/l/h (Gamal *et al.*, 2013).

TABLE 7. Bioethanol production by baker's yeast in continuous culture at 0.03 h^{-1} dilution rate (150 ml filtered hydrolysate* / h flow rate / 5000 ml culture).

Time (days)	Sugars in put (g/h)	Sugars out let (g/h)	Consumed sugars (g/h)	Bioethanol concentration out let (% v/v)	Bioethanol concentration out let (ml/h)	Conversion coefficient (% v/w)	Bioethanol yield (% v/w)	Productivity (ml/l/h)
1	1.665	0.007	1.658	0.49	0.735	29.4	29.6	0.02
2	1.665	0.008	1.657	0.51	0.765	30.6	30.8	0.021
3	1.665	0.01	1.655	0.52	0.78	31.2	31.4	0.022
4	1.665	0.01	1.655	0.52	0.78	31.2	31.4	0.022
Means	1.665	0.0088	1.656	0.51	0.765	30.6	30.8	0.021

*Filtered hydrolysed = obtained by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw in bioreactor.
The values are mean of three samples determinations. Standard deviation was within 5 %.
Conversion coefficient (%) = amount of bioethanol produced (% v/v) x 100 / sugars consumed (% w/v) (Gamal *et al.*, 2013).
Bioethanol yield (%) = amount of bioethanol produced (% v/v) x 100 / initial sugars concentration (% w/v) (Gamal *et al.*, 2013).
Productivity (P) = amount of bioethanol produced (% v/v) / fermentation time (h) = ml/l/h (Gamal *et al.*, 2013).

TABLE 8. Bioethanol production by baker's yeast in continuous culture at 0.04 h^{-1} dilution rate (200 ml filtered hydrolysate* / h flow rate / 5000 ml culture).

Time (days)	Sugars in put (g/h)	Sugars out let (g/h)	Consumed sugars (g/h)	Bioethanol concentration out let (% v/v)	Bioethanol concentration out let (ml/h)	Conversion coefficient (% v/w)	Bioethanol yield (% v/w)	Productivity (ml/l/h)
1	2.22	0.6	1.62	0.19	0.38	11.7	8.5	0.008
2	2.22	1.04	1.18	0.14	0.28	11.9	6.3	0.006
3	2.22	1.62	0.6	0.06	0.12	10.0	2.7	0.003
4	2.22	2.08	0.14	0.01	0.02	7.14	0.4	0.0004
Means	2.22	1.335	0.885	0.1	0.2	10.185	4.475	0.00435

*Filtered hydrolysate = obtained by the growth of *T. viride* EMCC 107 on 5 % w/v rice straw in bioreactor.

The values are mean of three samples determinations. Standard deviation was within 5 %.

Conversion coefficient (%) = amount of bioethanol produced (% v/v) x 100 / sugars consumed (% w/v) (Gamal *et al.,* 2013).

Bioethanol yield (%) = amount of bioethanol produced (% v/v) x 100 / initial sugars concentration (% w/v) (Gamal *et al.,* 2013).

Productivity (P) = amount of bioethanol produced (% v/v) / fermentation time (h) = ml/l/h (Gamal *et al.,* 2013).

By comparing bioethanol formation parameters obtained by baker's yeast from biotreated rice straw by different fermentation techniques conducted in this investigation, it could be concluded that the highest bioethanol concentration and productivity was attained in continuous culture technique at 0.03 h^{-1} dilution rate (0.51 % v/v and 0.021 ml/l/h, respectively). While the maximum amount of bioethanol obtained by using fed-batch technique, was by the continuous addition of filtered rice straw hydrolysate at specific addition rate of 151.5 ml/h (0.45 % v/v) after 30 h, followed by that obtained in pulsed fed-batch culture technique (0.41 % v/v) after 36 h. Regarding the high productivity of continuous culture technique at 0.03 h^{-1} dilution rate, this method was considered as the most efficient for bioethanol production by baker's yeast on biodegraded rice straw using *T. viride* EMCC 107. In a related investigation, continuous bioethanol production was carried out using *Sacch. cerevisiae* and a medium containing 280 g/l glucose. An average bioethanol concentration of 124.6 g/l or 15.8 % (v) was produced when the bioreactor system was operated at a dilution rate of 0.012 h^{-1}. The yield of bioethanol to glucose consumed was calculated to be 0.484 (Bai *et al.,* 2004). Saha & Cotta (2011) produced bioethanol continuously with high productivity from alkaline peroxide pretreated and enzymatically saccharified wheat straw hydrolysate under various conditions at controlled pH 6.5 and 35°C. The average bioethanol produced from the available sugars (21.9 – 47.8 g/l) ranged from 8.8 to 17.3 g/l (0.28 – 0.45 g/g available sugars, 0.31 – 0.48 g/g sugars consumed) with bioethanol productivity of 0.27 – 0.78 g/l/h in a set of 14 continuous culture runs (16 –105 days). Whereas, encapsulated *Sacch. cerevisiae* was able to ferment dilute acid lignocellulosic hydrolysate in continuous culture to bioethanol at dilution rates up to 0.5 h^{-1} with a bioethanol yield of 0.44 g/g and a specific productivity of 0.14 – 0.17 g/g/h (Talebnia

et al., 2006). Ali (2013) carried out continuous bioethanol production by *Sacch. cerevisiae* in bioreactor. The conditions that gave the best bioethanol productivity (7.57 g/l/h) was found to be an initial date syrup sugar concentration of 5.5 % (w/v) and a feeding flow rate of 294 ml/h.

Conclusion

In this study, the bioconversion of some lignocellulosic feedstocks to bioethanol was investigated. Our results revealed that fungal biotreatment of these pretreated feedstocks, especially rice straw, and the subsequent fermentation of obtained hydrolysates by baker's yeast had promising effects on increasing the overall bioethanol concentration. This research might play important role in bioethanol production and leave the door open on using some other feedstocks in studies similar to those conducted with rice straw. These data will also be helpful to construct a new approach for lignocellulosic bioethanol production in the future.

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انتاج الأيثانول الحيوى من بعض المخلفات الزراعية و الصناعية المعاملة بالفطريات

طارق سعيد الطيب ، أحمد عبد الوهاب عبد الحافظ ، صفوت حسن على[*] و الشحات محمد رمضان[**]
قسم الميكروبيولوجيا الزراعية ، [*]قسم الكيمياء الزراعية – كلية الزراعة – جامعة عين شمس – شبرا الخيمة و [**]معمل البيوتكنولوجى – جامعة هليوبوليس – طريق القاهرة – بلبيس الصحراوى – القاهرة – مصر .

يعتبر انتاج الأيثانول الحيوي من المخلفات اللجنوسيليلوزية احد الاستراتيجيات الواعدة لانتاج الايثانول كوقود حيوى من مصادر بديلة عن تلك المستخدمة كغذاء للانسان او الحيوان. تهدف هذه الدراسة لتقييم انتاج الايثانول الحيوى باستخدام خميرة الخباز على بيئة تحتوى على ناتج تحلل خمسة من المخلفات الصناعية الزراعية بواسطة الفطريات المحللة للسيليلوز مع بعض الاضافات. كما تم دراسة معاملة المخلفات اللجنوسيليلوزية بواسطة هيدروكسيد الصوديوم (5٪ حجمية/حجمية) و حامض كبيريتيك (1٪ حجمية/حجمية) و صوديوم هيبوكلوريت : فوق اكسيد الهيدروجين 1:10 قبل المعاملة الفطرية. لأنتاج الأيثانول فى المخمر و تم اجراء تنمية لخميرة الخباز على ناتج تحلل قش الأرز بالفطريات و ذلك بنظام الدفعة الواحدة و الدفعة الواحدة المغذاه و بالتنمية المستمرة. أعلى تركيز ناتج من الأيثانول و معامل تحويل للايثانول و محصول للايثانول و انتاجية و عند الانتاج بنظام الدفعة الواحدة و الدفعة الواحدة المغذاه كان 0,41 ٪ حجمية/حجمية ، 36,9 ٪ حجمية/وزنية ، 36,9 ٪ حجمية/وزنية و 0,114 مل/لتر/ساعة و على الترتيب و فى حين سجلت هذه التقديرات فى حالة التنمية بنظام الدفعة الواحدة المغذاه بالدفع المستمر (0,45 ٪ حجمية/حجمية ، 40 ٪ حجمية/وزنية ، 40,5 ٪ حجمية/وزنية و 0,015 مل/لتر/ساعة) و على الترتيب. تم الحصول على اعلى تركيز من الأيثانول (0,52 ٪ حجمية/حجمية) عند الانتاج بنظام التنمية المستمرة عند تخفيف 0,03 / ساعة ، حيث كان معامل التحويل للايثانول و محصول الايثانول و الانتاجية ، 31,2 ٪ حجمية/وزنية ، 31,4 ٪ حجمية/وزنية و 0,022 مل/لتر/ساعة , على الترتيب.