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Enhanced ethanol and glucosamine production from rice husk by NAOH pretreatment and fermentation by fungus *Mucor hiemalis*

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HIGHLIGHTS

- Ethanol production from rice husk using *Mucor hiemalis* was investigated.
- The maximum ethanol production yield of 86.7% was observed after pretreatment with 2.6 M NaOH at 67°C for 150 min.
- A highly valuable fungal biomass containing 60 g/kg glucosamine was obtained at optimum conditions.

GRAPHICAL ABSTRACT

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ABSTRACT

Ethanol production from rice husk by simultaneous saccharification and fermentation using *Mucor hiemalis* was investigated. To reach the maximum ethanol production yield, the most important influencing factors in the pretreatment process, including temperature (0-100°C), NaOH concentration (1-3 M), and the pretreatment time (30-180 min), were optimized using an experimental design by a response surface methodology (RSM). The maximum ethanol production yield of 86.7 % was obtained after fungal cultivation on the husk pretreated with 2.6 M NaOH at 67°C for 150 min. This was higher than the yield of 57.7% obtained using *Saccharomyces cerevisiae* as control. Furthermore, fermentation using *M. hiemalis* under the optimum conditions led to the production of a highly valuable fungal biomass, containing 60 g glucosamine (GlcN), 410 g protein, and 160 g fungal oil per each kg of the fungal biomass.

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1. Introduction

Due to the increasing global population, energy consumption has also increased, and the present fossil fuel resources do not seem to be able to meet the future energy demands in an environmentally-sustainable manner. To address this challenge, biofuels, as renewable, sustainable, and efficient energy resources, have been considered as promising resources (Zabochnicka-Świątek and Sławik, 2010). Biofuels, e.g., biogas, biodiesel, ethanol, and hydrogen produced from biomass, are suggested to be suitable future alterative energy resources. Among liquid biofuels, ethanol, as a clean and renewable energy source, has received significant deal of attention (Saha and Cotta, 2008; Karimi and Chisti, 2015). Ethanol can be produced from different raw materials, including sugar-based, starched-based, and lignocellulosic materials (Balat, 2011). Using inexpensive raw materials is crucial for the economically-feasible production of ethanol (Brethauer and Wyman, 2010). Lignocellulosic substrates are the most abundant renewable and inexpensive resources on the Earth.

It has been frequently reported that agricultural and forest residues are largely unused and available in huge capacity (Kim and Dale, 2004; Limayem and Ricke, 2012; Salehian and Karimi, 2013). For instance, rice is a plant that is widely cultivated in Asia, Africa, and Latin America. The cultivation of rice leads to the production of huge amounts of straw and husk. The husk yield is around 20% of the rice weight, which is mainly unused (Taherzadeh and Karimi, 2007). Lignin, cellulose, and hemicelluloses are the major constituents of lignocellulosic materials. Cellulose can be enzymatically hydrolyzed to glucose and then fermented to ethanol (Karimi and Pandey, 2014). However, the presence of lignin and hemicellulose decreases the access of hydrolytic enzymes to cellulose. To increase the enzymes' accessibility, lignin and hemicelluloses should be removed, and the complex structure should be opened up (Taherzadeh and Karimi, 2007). Thus, a pretreatment process is an essential and the most important step in biofuel production from lignocellulosic materials. An effective pretreatment should improve the hydrolysis while least sugar degradation is caused. Several pretreatment methods have been developed such as alkaline and acidic techniques (Taherzadeh and Karimi, 2008; Tabil et al., 2011). The alkaline pretreatments, e.g., by using NaOH, are among the most effective pretreatments (Shafiei et al., 2015).

After pretreatment, the pretreated materials can be either separately hydrolyzed and fermented or simultaneously hydrolyzed and fermented. Simultaneous saccharification and fermentation (SSF) is preferred, as the concentration of the released sugar is low, because the released sugars are directly consumed by the microorganisms. This reduces the risk of contamination, and it is also possible to conduct the process with lower enzyme loadings (Wyman, 1994). *Saccharomyces cerevisiae* is the most common organism used for the fermentation of hexose, while zygomycetes fungi can utilize a wider range of monosaccharides, including pentoses, and are of high performance for ethanol production from lignocellulosic materials (Sues et al., 2005). Furthermore, the biomass of these fungi are valuable products with various applications, due to the fact the it contains considerable amounts of glucosamine (GlcN) and essential fatty acids (Chatterjee et al., 2005; Bellou et al., 2012). Glucosamine, an amino monosaccharide, is a major component of the cell wall. Glucosamine has numerous applications, e.g., as a drug for osteoarthritis therapy and as a dietary supplement (Sitanggang et al., 2012; Mohammadi et al., 2013).

Recently, several researchers have studied the effect of NaOH pretreatment on enzymatic hydrolysis and ethanol production from lignocelluloses. Ko et al. (2009) investigated the effect of NaOH pretreatment on rice straw. The highest enzymatic digestibility of 71.1% was obtained at 69°C after 10 h pretreatment with 21% NaOH. In another study by Salehian and Karimi (2013), 8.0% NaOH solution was evaluated for the improvement of enzymatic hydrolysis from different parts of pine tree wastes. The high temperature pretreatment was shown to be more effective on the enzymatic hydrolysis, especially on needle leaves. In a different investigation, Cabrera-Rodríquez et al. (2013) reported the effect of NaOH pretreatment at low temperature on the chemical composition and enzymatic hydrolysis of spruce. The results showed changes in the material composition without significant carbohydrate hydrolysis. The yield of enzymatic hydrolysis was improved to 40% glucose. They concluded that alkaline pretreatment with NaOH is among the best and economical pretreatment methods.

In addition, there are some investigations on the effect of various pretreatments on rice husks. For example, Saha and Cotta (2008) optimized the pretreatment condition of rice hulls with lime. At the optimum conditions, i.e., 0.1 g lime/g hulls at 121°C for 1 h, the enzymatic saccharification yield of 32% was obtained. On the other hand, López et al. (2010) used dilute sulfuric acid pretreatment at high temperatures (160 to 210°C) for the production of fermentable sugars. The major problem with this process was production of fermentation by-products, including furfural and HMF. However, to the best of our knowledge, there is no investigation on the alkali pretreatment of rice husk and production of ethanol using zygomycetes fungi.

Therefore, in the current study, the most important affecting parameters during sodium hydroxide pretreatment, i.e., NaOH concentration, temperature, and time, were optimized to improve the ethanol production by zygomycetes fungus *Mucor hiemalis*. Besides, the production of other valuable fermentation by-products was also investigated.

2. Materials and Methods

2.1. Substrate, microorganism, and enzymes

Rice husk from a cultivar named Sazandegi was obtained from Zarinshahr (Isfahan, Iran). It was ground and screened to achieve particles with less than 0.8 mm.

M. hiemalis CCUG 16148, obtained from Collection University of Gothenburg, Sweden, was used for efficient fermentation. The fungal spores were cultivated on agar slants, containing (g/L): glucose (40), agar (20), and peptone (10) at 32°C for 5 d. Besides, *S. cerevisiae* CCUG 53310 form the same culture collection was used for comparison purpose. The yeast medium was prepared according to Karimi et al. (2006). The slants were stored at 4°C until use.

Two commercial enzymes, cellulases (Celluclast 1.5 L, Novozyme, Denmark) and β-glucosidase (Novozyme 188, Novozyme, Denmark), kindly provided by Novozymes, were used for hydrolysis of cellulose.

2.2. Pretreatment

Five g of rice husk (based on dry weight) was mixed with 95 g of NaOH. The pretreatments were conducted with 1-3 M NaOH at different temperatures in the range of 0-100 °C for different times in the range of 30-180 min, as suggested by the experimental design. Statistically based experimental designs were applied to optimize the pretreatment conditions. Afterward, the pretreated solids were washed several times with distilled water, dried at 32°C to achieve a constant weight, and stored at room temperature. The carbohydrates and lignin (acid-soluble and insoluble) contents of untreated and pretreated rice husk were analyzed using the standard method presented by Sluiter et al. (2012).

2.3. Production of ethanol by simultaneous saccharification and fermentation (SSF)

Optimization of variables (temperature, time, and NaOH concentration) was conducted with response surface methodology (RSM) by central composite design (CCD) using Design Expert 7.0.0 software. To determine the optimum pretreatment conditions, the ethanol yield (g/g initial sugar) was considered as the response. The combined effect of variables was studied on ethanol production and hydrolysis yields with CCD. Suggested by the design, 20 experiments were performed.

For ethanol production using SSF, 1.0 g pretreated or untreated rice husk and 20 ml nutrient solution containing (g/L): yeast extract, 5.0; $(NH_4)_2SO_4$, 7.5; $MgSO_4 \cdot 7H_2O$, 0.75; KH_2PO_4, 3.5; and $CaCl_2 \cdot 2H_2O$, 1.0 in 0.05 M sodium citrate buffer (pH 4.8) were added to a 118 ml bottle. The suspension was then autoclaved for 20 min at 121°C. After cooling to room temperature, the solutions were inoculated with 100 µL of a suspension containing 3.8 (±0.6) $\times 10^5$ spores/mL of *M. hiemalis*, and then supplemented with 30 FPU cellulase and 60 IU β-glucosidase per gram of dry substrate. Finally, the bottle was purged with oxygen-free nitrogen and incubated at 37°C and 120 rpm for 72 h. Liquid samples were periodically taken and analyzed by high-performance liquid

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chromatography (HPLC) for sugar and ethanol analyses. All experiments were conducted in duplicates.

2.4. Production of biomass

Biomass was produced with separate hydrolysis and fermentation (SHF). The hydrolysate was produced by enzymatic hydrolysis at 45°C in 0.05 M sodium citrate buffer. The substrate concentration was 50 g/L (based on the dry weight of the pretreated samples) and the initial pH was adjusted to 4.8. The suspension was autoclaved at 121°C for 20 min. After cooling to room temperature, 30 FPU cellulose and 60 IU β-glucosidase per gram of dry substrates were added. The hydrolysis was performed for 72 h at 45°C and 120 rpm. A glucose assay kit was used to measure the glucose content in the samples (Bondar and Mead, 1974). Afterwards, the hydrolysates and glucose (as a reference) solutions were supplemented with the required nutrients including (g/L): yeast extract, 5.0; $(NH_4)_2SO_4$, 7.5; $MgSO_4{\cdot}7H_2O$, 0.75; KH_2PO_4, 3.5; and $CaCl_2{\cdot}2H_2O$, 1.0. The initial pH was adjusted to 5.5 ± 0.1, and the media were autoclaved at 121ºC for 20 min. After cooling to room temperature, the suspensions were inoculated with 1.0 mL of a suspension containing 3.8(±0.6) $\times 10^5$ *M. hiemalis* spores/mL or a loop full of *S. cerevisiae*. Each media was purged with oxygen-free nitrogen to provide anaerobic conditions and then incubated at 32 ºC and 120 rpm for 48 h. At the end of fermentation, ethanol content of liquid medium was determined by HPLC and the biomass was separated by centrifugation, washed with distilled water, freeze-dried, and stored for further analyses.

2.5. Analytical method

Protein and lipid contents of the biomass were analyzed according to the Biuret (Verduyn et al., 1990) and Debjani et al. (2012) methods, respectively. The glucosamine content was investigated according to the procedure described by Mohammadi et al. (2012). To obtain the cell wall materials (i.e., alkali insoluble material - AIM), the dried biomass was treated with 2% NaOH solution at 120ºC for 20 min. The AIM of biomass was collected after centrifugation at 4000 rpm for 15 min, washed with distilled water to obtain neutral pH, and centrifuged.

The amounts of sugars and ethanol were analyzed by a HPLC equipped with UV-vis and RI detectors (Jasco International Co., Tokyo, Japan). For sugar analysis, an ion-exchange Aminex HPX-87P column (Bio-Rad) was used at 85°C with deionized water as eluent at a flow rate of 0.6 mL/min. Ethanol was analyzed using Aminex HPX-87H column (Bio-Rad) at 60°C with 5 mM sulfuric acid as eluent at a flow rate of 0.6 mL/min.

Scanning electron microscopy (SEM) was used to study the effects of pretreatment on the structure of the best pretreated and untreated rice husk samples. Dried treated and untreated straw samples were coated with gold (BAL-TEC SCD 005), and images were recorded at 15 kV by a SEM (PHILIPS, XL30).

The crystallinity of cellulose in the untreated and pretreated husk, at which the highest hydrolysis was observed, was determined using Fourier Transform Infrared (FTIR) spectrometry equipped with a universal Attenuated Total Reflection (ATR) accessory and Deuterated triglycine sulfate (DTGS) detector (Bruker Tensor 27 FT-IR). The spectra were acquired at 4 /cm resolution with an accumulation of 60 scans per sample, recorded in the range of 600–4000 /cm, normalized to the highest peak, and used for calculating the crystallinity.

3. Results and Discussion

3.1. Pretreatment conditions

The rice husk was pretreated with 1-3 M NaOH at temperatures 0 - 100°C for 30-180 min (**Table 1**). The low and high values for each factor were selected according to preliminary experiments and the literature (Tabil et al., 2011; Cabrera-Rodríquez et al., 2013; Salehian and Karimi, 2013).

Table 1.
Factors of RSM experimental design.

Factor	Indicator	High level	Low level
Temperature (˚C)	A	0	100
Time (min)	B	30	180
NaOH concentration (M)	C	1	3

3.2. Effects of pretreatment on rice husk composition

The composition of the rice husk was determined before and after the pretreatments. As shown in **Table 2**, the rice husk contained (%): glucan, 36.2; xylan, 15; lignin, 19.8; and ash, 18.6. The untreated rice husk contained 52.8 % carbohydrates and 38.4 % lignin. The pretreatments increased the carbohydrates fraction of the pretreated samples to 55.6−62.4%. This was due to the fact that the lignin and ash contents were decreased by the pretreatments. These results were in line with those of the previous studies (Saha and Cotta, 2008; Salehi et al., 2012; Khaleghian et al., 2015).

3.3. Optimum pretreatment conditions

The design matrix and the results are shown in **Table 3**. A second-order polynomial was established using the Design Expert software to identify the relationship between the ethanol yield and the three significant

Table 2.
Chemical composition (wt. %) of the untreated and pretreated rice husk.

Pretreatment conditions			Components (%)*				
T (°C)	Time (min)	NaOH Concentration (M)	Glucan	Xylan	Mannan	Lignin	Ash
50	180	2	43.1	12.2	2.8	15.8	16.5
20	150	2.6	39.7	14	1.9	17.3	17
20	60	2.6	41.7	14.6	1.9	15.2	16.9
50	105	3	42.9	12.2	2.9	15.7	15.6
50	105	2	45.7	12.6	2.2	14.3	15
50	105	1	40.7	12.7	2.3	17.7	16
80	60	2.6	39.9	13.8	2.5	17.9	16.3
0	105	2	39	14.8	2.2	17.7	17.9
100	105	2	45.6	12.1	3.8	15	14.2
80	150	2.6	47.3	13	2.1	13.6	14.1
20	150	1.4	39.8	14.3	2.8	16.23	17.2
20	60	1.4	39.9	14.7	2.3	16.76	17.46
80	150	1.4	43.7	12.8	1.8	15.9	15.6
80	60	1.4	44.7	13	1.7	15.4	14.9
50	30	2	39.4	14.3	2	17.4	17
Untreated rice husk			36.23	15	1.55	19.8	18.6

* S.D. of all duplicated data was less than 4.8%.

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variables as follows (**Eq. 1**):

$$\text{Ethanol concentration} = +10.06+0.73\times A+0.73\times B+0.69\times C-0.32\times A\times B+1.68\times A\times C+0.9\times B\times C-1.16\times A^2-1.16\times B^2-1.02\times C^2 \quad (1)$$

where A, B, and C are the concentration of sodium hydroxide solution, temperature, and pretreatment time, respectively.

Table 3.
RSM experimental design and the results of the produced ethanol.

Run	A	B	C	Ethanol yield (%)*
1	50	180	2	67.8
2	20	150	2.6	66
3	20	60	2.6	54.2
4	50	105	3	75.4
5	50	105	2	76.9
6	50	105	2	77.3
7	50	105	2	78
8	50	105	2	79.8
9	50	105	1	42.1
10	20	150	1.4	43.8
11	50	30	2	58.7
12	20	60	1.4	56.2
13	80	60	1.4	62.3
14	100	105	2	72
15	80	150	1.4	47
16	50	105	2	77
17	0	105	2	43.8
18	80	60	2.6	20.9
19	50	105	2	77.1
20	80	150	2.6	85.2

* S.D. of all duplicated data were less than 4.1%.

Contour and surface plots shown in **Figures 1, 2, and 3**, present the effect of temperature and time, temperature and NaOH concentration, and NaOH concentration and time on ethanol production simultaneously. According to **Figure 1**, the ethanol yield increased with increasing time and temperature. However, at temperatures higher than 100ºC, the yield of ethanol decreased even by increasing time. **Figure 2** shows the positive effect of the NaOH concentration on the yield of ethanol. Ethanol production was increased with increasing the NaOH concentrationn and time. However, the yield of ethanol

Fig.1. Contour and surface plots of the interaction between time and temperature on ethanol concentration.

Fig.2. Contour and surface plots of the interaction between time and NaOH concentration on ethanol concentration.

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increased with increasing NaOH concentration and temperature up to a certain level and then decreased. Thus, temperature was the limiting factor and the optimum range of temperature for the alkali pretreatment of rice husk with NaOH was 50 - 80ºC. According to the model, the optimum condition was pretreatment with 2.6 M NaOH at 67ºC and for 150 min. This was similar to the results obtained by Ko et al. (2009), in which the best enzymatic digestibility of rice straw was observed after pretreatment with NaOH at 69ºC.

Fig.3. Contour and surface plots of the interaction between NaOH concentration and temperature on ethanol concentration.

3.4. Effect of pretreatment on ethanol production at optimum conditions

The results showed the ethanol yield of 15.1 and 86.7% for the untreated and the pretreated rice husk samples under the optimum conditions, respectively. The results were close to the results obtained by Saha and Cotta (2008) who produced 0.43 g ethanol/g available sugars using dilute acid pretreatments of the rice hull and fermentation using *Escherichia coli*. Furthermore, for comparison purposes, ethanol was produced in the present study at the optimum conditions by *S. cerevisiae*. The ethanol yield stood at 10.1 and 57.7% for the untreated and treated rice husk, respectively. Thus, *M. hiemalis* was shown to have a better performance than *S. cerevisiae*. This may be related to the high resistance of the fungus against the inhibitors as well as consumption of pentoses (Nag, 2008). These results were also comparable with the results obtained by Khaleghian et al. (2015). They also reported a better performance of *M. hiemalis* compared with *S. cerevisiae* in terms of ethanol production from rice straw pretreated with Na_2CO_3.

The results obtained through the present study were encouraging in terms of product yield and volumetric ethanol productivity using *M. hiemalis* for further scale-up studies and commercial exploitation.

3.5. Effect of pretreatment on cellulose crystallinity

Crystallinity index (CI), the absorbance ratio of A1430/A896, was determined using FTIR spectra (**Fig. 4**). The peaks at 896 and 1430 /cm denote cellulose I and cellulose II, respectively (Salehi et al., 2012). Thus, CI changes under different pretreatments implied chemical changes in the structure of rice husk. The CI was 1.05 and 0.54 for the untreated and sodium hydroxide-treated husk samples under the optimum conditions, respectively. The results obtained indicated a significant CI reduction by sodium hydroxide pretreatment. This may be due to the decrease of some crystal regions of cellulose that caused the increase in the hydrolysis yield. These findings were similar with those reported by Goshadrou et al. (2011). They studied the effect of pretreatment with NaOH on the CI of sweet sorghum and showed a reduction in CI from 0.83 to 0.73 after pretreatment with 12% NaOH at 0ºC for 3h. In another study, Nieves et al. (2011) reduced the CI of oil palm empty fruit bunches (OPEFB), a waste lignocellulosic material, from 1.05 to 0.46 after pretreatment with NaOH 8% for 60 min.

Fig.4. FTIR spectra of (1) untreated rice husk and (2) those pretreated with NaOH 2.6 M at 67°C for 150 min.

3.6. Effect of pretreatment on rice husk morphology

The SEM images showed significant morphological modifications of the pretreated husk (**Fig. 5 a, b**). The untreated substrate had a compact and inaccessible structure with a negligible porosity. Sodium hydroxide basically disrupted the structure of the fibers, and sponge-like structures were observed after the pretreatment with NaOH. This could provide a higher surface area for the enzymatic reactions. Sarkar et al. (2012) also showed an increased porosity and disruption of the structure of rice straw after the alkaline pretreatment.

3.7. Protein, lipid, and glucosamine production by M. hiemalis

The biomass of the *M. hiemalis* grown on the hydrolysate obtained after the optimum pretreatment conditions was analyzed. The major ingredients detected were protein (41%), lipid (16%), and AIM (13%). Chatterjee et al. (2008) claimed 37.5% protein content in *Rhizopus oryzae* biomass which was almost similar to that obtained using the fungus studied in the present study.

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Fig.5. SEM images of rice husk before and after pretreatment: (a) untreated and (b) pretreated with NaOH 2.6 M at 67ºC for 150 min.

Lipids produced by oleaginous fungi are the focus of a number of recent investigations, as they are highly valuable nutritionally (Dey et al., 2014). The production of lipid from *M. hiemalis* was not previously recorded in the literature. The amount of lipid in biomass was less than that obtained by Debjani et al. (2012) for *Mucor circinelloides* (46%).

The amount of AIM in zygomycetes is reportedly in the range of 13-19.3% (Zamani et al., 2007; Zamani et al., 2010), and the fungus used in this study contained 13% AIM, i.e., on the lower border of the range.

The glucosamine content was recorded at 0.46 g/g AIM. The amount of glucosamine was reported between 0.37-0.47 g/g AIM for different zygomycetes fungi (Zamani et al., 2010). Thus, the biomass obtained herein was highly rich in glucosamine, which is a valuable byproduct that could improve the economy of ethanol production.

4. Conclusions

The native form of rice husk is not a suitable substrate for ethanol production, and therefore, its ethanol production yield was recorded at only 15.1%. However, a high yield of 86.7% could be obtained when the husk was pretreated with NaOH. The pretreatment could remove the lignin, modify the structure, and decreased the cellulose crystallinity. Besides, fermentation using *M. hiemalis* led to the production of appreciable amounts of value-added products, including glucosamine, protein, and lipid.

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