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Response surface methodology based optimization studies about bioethanol production by *Candida boidinii* from pumpkin residues

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Abstract

For sustainable bioethanol production, the investigation of novel fermentative microorganisms and feedstocks is crucial. In this context, the goals of the current study are suggesting pumpkin residues as new raw material for bioethanol production and investigating the fermentative capacity of the *Candida boidinii*, which is a newly isolated yeast from sugar factory wastes. Response surface methodology was used to determine the effect of enzyme (cellulase and hemicellulase) concentration and enzymatic hydrolysis time. The maximum bioethanol concentration was 29.19 g/L when fermentation parameters were optimized. However, it is revealed that enzymatic hydrolysis and hydrolysis duration (48-72 h) have significant effects on reducing sugar concentration. The highest reducing sugar was 108.86 g/L when the 20% initial pumpkin residue was hydrolyzed at 37.5 FPU/g substrate cellulase and 37.5 U/mL hemicellulase at the end of 72 h. Under these optimized conditions, the bioethanol production of *C. boidinii* increased by 22.91% and reached 35.88 g/L. This study shows pumpkin residues are promising feedstocks and *C. boidinii* is a suitable microorganism for efficient bioethanol production.

Introduction

Biofuels are sustainable, eco-friendly, and cheap alternatives to fossil fuels. Among them, bioethanol attracts attention because of its renewable and eco-friendly features (Nowicka et al., 2020). Furthermore, another usage area of bioethanol is the production of hand sanitizers or disinfectants which are very useful agents against pathogen microorganisms. Moreover, the COVID-19 outbreak caused a massive demand for alcohol-based disinfectants and ethanol shortage (Itiki & Chowdhury, 2020). For this reason, ethanol prices increased and ethanol production gained importance for public health. Therefore, the studies about bioethanol production gained importance in the literature (Mahlia et al., 2019; Palupi et al., 2020; Song et al., 2020).

Bioethanol is derived from biomass and this substance can be classified into different generations according to the type of raw materials used. The first-generation bioethanol is produced by sugar-containing feedstocks such as starch, maize, wheat, sugarcane, or sugar beet. Raw materials from the first generation have high productivity rates; however, they have a negative impact on food prices. On the other hand, the source of the second-generation bioethanol is lignocellulosic

feedstock which is one of the most abundant and cheap materials on earth (Adıgüzel, 2013). Third-generation bioethanol is obtained from photosynthetic microorganisms and genetically modified microorganisms are used for fourth-generation ethanol production.

Lignocellulose is the most abundant and under-utilized feedstock on Earth. Thus, it does not compete with edible sources for energy production and, does not affect the food production chain (Kumar et al., 2016; Naik et al., 2010). However, lignocellulosic bioethanol production is still problematic because of the recalcitrance of the raw materials (Paul & Dutta, 2018). Moreover, by-products that are generated from the pre-treatment of lignocellulose can reduce the activity of the enzymes or inhibit microbial growth (Aytaş et al., 2023). Therefore, the determination of the efficient bioethanol producer organisms or available feedstocks can contribute to more effective bioethanol production processes.

Efficient ethanol production from all sugars present in lignocellulosic raw material is crucial for more economical bioethanol production. Xylose is the second most abundant fermentable sugar in lignocellulose after glucose. Therefore, the utilization of xylose is of great importance for efficient fermentation. However, commercially available yeasts such as *Saccharomyces cerevisiae* cannot ferment xylose into ethanol (Zhao et al. 2016). For these reasons, it is vital to identify novel yeast strains which are able to ferment a broad range of sugars into ethanol. In this context, *C. boidinii* can be a good alternative to conventional ethanol producers such as *S. cerevisiae* because of its high acid tolerance and broad range of sugar utilization capacity (Osawa et al., 2009; Santana et al., 2018). However, despite its potential, studies on the bioethanol production from *C. boidiini* in the literature are very limited. For these reasons, in the current study, the bioethanol production of *C. boidinii* was compared to that of *S. cerevisiae* that is the most commonly used ethanol-producing microorganism for bioethanol production.

Investigation of food by-products which contain lignocellulosic biomass is an important step for environmental protection and bioethanol production (Schieber et al., 2001). Pumpkin residues (PR) are rich in carbohydrate, β-carotene, as well as cellulose and hemicelluose. For these reasons, the main objective of the current study is the evaluation of PR as a raw material for bioethanol production.

Response Surface Methodology (RSM), one of the statistical methods, is useful for making predictions that are more accurate and require less experimental datasets. When conventional methods are unable to identify the combined impacts of all the variables, the method also enables researchers to examine how different variables interact (Yolmeh & Jafari, 2017; Pereira et al., 2021). Due to its advantages, RSM is commonly used in lignocellulosic pre-treatment and bioethanol production research (Chen et al., 2020; Manmai et al., 2021). Therefore, we used the same trend and used RSM in the current study (Yolmeh & Jafari, 2017; Pereira et al., 2021). Due to its advantages, RSM is commonly used in lignocellulosic pre-treatment and bioethanol production research (Chen et al., 2020; Manmai et al., 2021). Therefore, we used the same trend and used RSM in the current study (Yolmeh & Jafari, 2017; Pereira et al; 2021). For the mentioned reasons, during lignocellulosic pre-treatment and bioethanol production studies RSM is widely applied (Chen et al, 2020; Manmai et al. 2021). Because of the mentioned reasons RSM was used for bioethanol production optimization in the current study.

In the first part of the study, bioethanol production, glucose and xylose assimilation capacities of the different yeasts were tested. After that step, fermentation conditions were optimized by RSM. During the experiments, enzymatic hydrolysis rate were increased with optimization and bioethanol production of the yeasts were monitored. According to the results, the novel isolate of *C. boidinii* produced more bioethanol than model microorganism *S. cerevisiae.* This isolate was also able to assimilate xylose as well as glucose. To the best of our knowledge, this is the first report about bioethanol production from pumpkin residues used by *C. boidinii*.

Materials, and Methods

Isolation, PCR and sequencing of yeast cells

The samples were collected from sugar factory waste and were used for isolation studies. These samples were centrifuged and spread (0.1 mL) on Petri plates containing Potato Dextrose Agar (PDA/Merck-Germany) media. PDA media was supplemented with 600.000 IU penicillin, and were incubated at 30 °C. Cells from microcolonies on these plates were isolated and purified by streaking the cells repeatedly on the PDA plates. The pure cultures were kept at +4 °C and were transferred to fresh PDA media periodically. Purified colonies were screened for their bioethanol production capacities. The cell that showed the most promising ethanol production capacity was identified. Sugar beet molasses medium was used for screening. For this purpose, 300 g/L sugar beet molasses was pre-treated with 1.5% H_2SO_4 (Merck-Germany), and autoclaved at 121 °C for 15 minutes (min). This stock medium was diluted to 8% (v/v) with sterile distilled water. pH was adjusted to 5 with 10 N NaOH (Merck-Germany). 1.0 g/L $(NH_4)_2SO_4$ (Merck-Germany) and 0.5 g/L KH_2PO_4 (Merck-Germany) were added to molasses medium and 1 g/L cells were inoculated to molasses medium. Incubation time was set to 30 °C.

ITS regions were amplified with ITS1 and ITS4 primers (Glass & Donaldson, 1995). DNA extraction was carried out with EurX GeneMATRIX Bacterial & Yeast DNA kit (Poland). Thermo Scientific Nanodrop 2000 (Massachusetts/USA) was used for calculations of DNA purity and concentration. PCR was conducted by initial

denaturation at 94 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 45 seconds (s). Annealing was performed at 57 °C for 45 s, and extension was carried out at 72 °C for 60 s. MAGBIO "HighPrep™ PCR Clean-up System" (AC-60005) were used for the PCR product was purification. ABI 3730XL (Applied Biosystems, Foster City, CA) with BigDye Terminator v3.1 Cycle (Applied Biosystems, Foster City, CA) sequencing kit was used for DNA was sequencing. Identification was performed by an external laboratory (Refgen, Ankara, Turkey).

Pre-treatment of PR

PR was collected from the local market in Ankara/Turkey. These PR were dried in an oven overnight at 70 °C (Nuve/Turkey), and the dried residues were grounded in laboratory type mill with a 0.1 cm mesh size screen, and kept in a screw cap bottle until used in the experiments.

1% H_2SO_4 (Merck-Germany) was used for pre-treatment experiments. PR was autoclaved in 121 °C (ALP/CL-40M/Germany) for 15 min immediately after acid pre-treatment. For the fermentation assays, this slurry was filtered through Whatman No.1 paper and used for fermentation experiments.

Enzymatic hydrolysis

Commercial cellulase CelliCTec2 (d: 1.15 g/mL, 121 FPU/mL, Sigma-Aldrich) and hemicellulase from *Aspergillus niger* (0.3-3.0 U/ mg solid, Sigma-Aldrich) were used for enzymatic hydrolysis. Cellulase concentration was adjusted to 15 FPU/g cellulose and hemicellulase loading was set to 15 U/mL. Enzymatic hydrolysis was carried out at 50 °C and pH 4.8 in the presence of 50 mM citrate buffer for 72 hours (h). Agitation speed was adjusted to 100 rpm (Chen et al., 2012).

Response surface methodology

To evaluate the effects of independent variables on the bioethanol production of the new isolate, The Design Expert Software program (StatEase®) was used for RSM (6 center points, 20 total run) was used. Total 20 runs were generated for RSM. Cellulase loading (15-60 FPU/g cellulose), hemicellulase loading (15-60 U/mL), and enzymatic hydrolysis time (24-72 h) were selected as independent factors for RSM experiments. 1% H_2SO_4 (v/v) pre-treatment was performed for all RSM experiments because of its low cost and effectiveness (Loow et al. 2016).

Fermentation experiments

The fermentation experiments were performed at 100 mL Erlenmeyer flasks with a working volume of 50 mL PR media. Incubation temperature was set to 30 °C for 96 h at 100 rpm agitation speed. PR media was supplemented with peptone (Merck-Germany/0.5 g/L), yeast extract ((Merck-Germany/3.0 g/L), $MgSO_4.7H_2O$ (Merck-Germany/0.5 g/L), KH_2PO_4 (Merck-Germany/1.0 g/L), $CaCl_2$ (Merck-Germany/0.1 g/L), and $ZnSO_4$ (Merck-Germany/0.05 g/L).

Analytical methods

Ethanol content was measured with gas chromatography (GC), (GC2010/Shimadzu/Japan). Before GC analysis, 1.5 mL of samples were centrifuged at 10000 rpm for 10 min (Hettich/320R/Germany). The supernatant was filtered through 0.22 µm membrane filter, and 1 µL of sample was injected through the SPL unit. The Restek Rtx-Wax column (60 m length, 0.25 mm ID.) and flame ionization detector (FID) were used for ethanol detection. The temperature of the injection port, and detector were set at 140 ºC and 160 ºC, respectively. The initial column temperature was 50 ºC, and the column temperature was increased to 150 ºC within 19 min. Column flow was 1.86 mL/min, and nitrogen was used as a carrier gas (Wistara et al., 2016).

The HPLC (Shimadzu/Japan) system with Coregel 87H3 (Transgenomic/USA) column and refractive index detector (RID10A) were used for the detection of the sugars, 5-hydroxymethylfurfural (HMF), acetic acid and formic acid present in PR. Before the analysis, 1.5 mL of samples were centrifuged at 10000 rpm for 10 min. The supernatant was filtered through 0.22 µM membrane filter. The column oven temperature was held at 70 ºC, and the total flow was set at 0.5 mL/min. 5 mM H_2SO_4 was used as a mobile phase. Samples were analysed for 25 min (Motoda et al., 2019).

Total reducing sugar was determined by the DNS method (Miller, 1959). Filter paperase unit (FPU) of the enzyme was determined according to Adney and Baker (2008). Theoretical ethanol yields were calculated according to Eq. (1) which was presented below (Kim & Lee, 2007).

Eq.(1):

$$Theoretical\ ethanol\ yield\ (\%) = \frac{ethanol\ (gL^{-1})}{initial\ sugar\ (gL^{-1}) \times 0.511} \times 100$$

Volumetric ethanol productivity (Qp) was calculated according to Eq. (2), as described as describe in the research published by (Roca & Olsson, 2003).

Eq. (2):

$$Q_p(g/Lh) = \frac{ethanol\ (gL^{-1})}{h_{\text{maximum}\ ethanol}}$$

Ethanol yields ($Y_{P/S}$) were determined according to Eq. (3) (Günan Yücel & Aksu, 2015):

Eq. (3):

$$Y_{P/S}\ (g/g) = \frac{maximum\ ethanol\ (gL^{-1})}{consumed\ sugar\ (gL^{-1})}$$

The cellulose concentration of raw PR was determined according to the standard ISO protocol (ISO 5498-1981). Cellulose determination was performed by an external laboratory, namely Düzen Norwest/Ankara.

Results and Discussion

Identification of yeast and effect of biomass loading and inhibitory compounds on bioethanol production

For sustainability, it is crucial to find and identify novel bioethanol-producer microorganisms which can

grow in lignocellulosic feedstocks. In this context, the isolation stage has vital importance. Therefore, the yeast which showed the highest growth in PR medium was selected and sequenced for identification.

Morphologically, yeast that used in the current study are approximately 0.05 mm, smooth, and form white colonies. Density of the cell is opaque and form of the colonies is circular. Microscopically, the cell shape was ellipsoidal budding. Whole cells from the exponentially growing culture of the isolate were used for internal transcribed spacers (ITS's). According to the sequencing results, the isolate showed 100% similarity with one *C. boidinii* strain (ON409985.1) and has more than 98% similarity with many other *C. boidinii* strains, such as UCDFST:09-399, CBS:6202 or CBS7299.

Initial biomass loading is an important parameter for the fermentation process. Moreover, higher initial biomass loadings are desirable since they result in higher sugar yields and lower production costs (Dutra et al., 2018). For these reasons, three different initial biomass loadings (10%, 20%, and 30% w/v) were tested in order to determine their effects on reducing sugar and ethanol concentrations. Results are presented in Table 1. It was observed that increased biomass loading caused higher sugar concentrations. The maximum sugar concentration was obtained from a 30% initial biomass loading at 78.32 g/L. On the other hand, 43.65 g/L and 59.80 g/L reducing sugar were found in 10% and 20% initial biomass loadings, respectively. These values are similar to those reported by Mithra and Padmaja (2017a), who obtained 40.56 g/L reducing sugar from 15% pumpkin peel when the biomass was pre-treated with 1% H_2SO_4 at 121 °C for 60 min.

During the experiments conducted with initial biomass loading, the ethanol concentrations of *C. boidinii* and *S. cerevisiae* were determined. Similar to reducing sugar concentrations, increased initial biomass loading resulted in increased ethanol production in both tested yeasts. There was no significant difference between the ethanol production of yeasts which are 21.00 g/L and 20.67 g/L respectively, at the end of 48 h fermentation time. Furthermore, ethanol concentration of *C. boidinii* increased to 26.57 g/L when the 30% initial biomass was used. At the same conditions, 24.58 g/L of ethanol was observed by *S. cerevisiae* (Table 1).

According to the results, the ethanol production capacity of *C. boidinii* was slightly higher than obtained from *S. cerevisiae,* which is the primary microorganism for commercial ethanol production. These results clearly indicate that *C. boidinii* is a promising agent for ethanol production. Therefore, *C. boidinii* was selected for further experiments in the current study.

Inhibitory compounds such as HMF, acetic acid, or formic acid have a negative effect on microbial growth and ethanol fermentation (Palmqvist & HahnHägerdal, 2000 kaynakçada yok). For these reasons, inhibitor concentrations of PR were also identified in this part of the study. The results in Table 1 demonstrate that higher biomass loading caused increasing in inhibitor concentrations. The highest acetic acid, and formic acid concentrations were detected as 0.81 g/L and 0.38 g/L, respectively, in the presence of 30% initial biomass loading. Nevertheless, these inhibitor concentrations are low in comparison with the literature (Parajó et al., 1998). Furthermore, much lower HMF concentrations were detected in comparison with acetic and formic acids. At 10% initial PR loading, 0.027 g/L HMF was obtained, and this value increased to 0.10 g/L when the initial PR loading adjusted to 30%. According to the report of Santana et al. (2018), *C. boidinii* metabolized more than 99% of the HMF present in the hemicellulolytic hydrolysate of non-detoxified cocoa pod husks. By this context, in the current study, mild pre-treatment conditions, low inhibitor concentrations of PR, and inhibitory tolerance of *C. boidinii* may have caused the higher bioethanol concentrations observed even in the presence of high PR loading.

Although the highest sugar and ethanol concentrations were observed in the presence of 30% (w/v) initial PR loading, due to the mass transfer limitations and water holding capacity of PR, further studies were carried out in the presence of 20% (w/v) initial PR loading.

Effects of enzymatic hydrolysis

Before enzymatic hydrolysis, 20% PR was pretreated with 1% H_2SO_4 for 15 min at 121 °C. The data in Figure 1 depicts that enzymatic hydrolysis of 20% PR loading caused higher sugar concentrations than 30% initial PR loading without enzymatic hydrolysis. 81.58 g/L the reducing sugar was obtained from 20% enzymatically hydrolyzed PR. A previous report in the literature showed that dilute acid pre-treatment and enzymatic hydrolysis of 10% PR resulted in 52.47 g/L reducing sugar (Mithra & Padmaja, 2017b).

In the current study, *C. boidinii* and *S. cerevisiae* produced similar ethanol concentrations from PR. However, the highest ethanol concentration was found to be 29.19 g/L from *C. boidinii* at the end of the 48 h fermentation time. *S. cerevisiae* produced 26.92 g/L ethanol (Figure 1). These values are higher than the report of Gonçalves et al. (2013), who used *C. boidinii* UFMG14 and found 12 g/L ethanol at the end of the same fermentation period from the hemicellulosic hydrolysate of macauba presscake. Furthermore, in the current study, after 48 h of fermentation, the ethanol concentrations of *C. boidinii* and *S. cerevisiae* declined to 24.43 g/L and 18.70 g/L, respectively. This decline may be related to assimilating of accumulated ethanol. Similar assimilation patterns were also reported previously from *C. boidinii* (Vandeska et al., 1995) and *Pichia stipitis* (Huang et al., 2009). Moreover, a significant ethanol production difference was observed between two yeasts in the early stages of fermentation. For instance, *C. boidinii* and *S. cerevisiae* produced 8.17 g/L and 21.44 g/L of ethanol in 18 hours and 11.09 g/L and 24.24 g/L of ethanol in 24 hours, respectively (Figure 1). This difference can be explained by the Crabtree

effect. In Crabtree-positive yeasts such as *S. cerevisiae*, alcoholic fermentation can be initiated when aerobic and sugar-limited cultures are exposed to sugar excess. On the other hand, this instantaneous response is not observed in Crabtree-negative yeasts, such as *C. boidinii* (Osawa et al., 2009). Therefore, prolonged bioethanol production period can be attributed to Crabtree-negative nature of *C. boidinii*.

Kinetic parameters for bioethanol production belonging to both yeasts were given in Table 2, and kinetic parameters of *C. boidinii* were higher than those of *S. cerevisiae*. The highest theoretical ethanol yield from *C. boidinii* was 70.0%. On the other hand, *S. cerevisiae* reached 64.5% of the theoretical ethanol yields. At the end of the 48-hour fermentation period, *C. boidinii* and *S. cerevisiae* generated 0.60 and 0.56 g/L.h. of ethanol, respectively. The respective ethanol yields of these yeasts were 0.46 and 0.42 g/g. These values are higher than when NaOH and ammonia conditioned rice straw was used in the literature (Lin et al., 2012). The reason of higher yields may be the efficient recovery of the xylose after dilute acid pretreatment.

Response surface methodology

Descriptive table of the independent variables and response belong to the RSM are shown in Table 3. The results of experimental runs with three independent variables (cellulase loading, hemicellulase loading, and hydrolysis time) and response (reducing sugar) are given in Table 4. All experiments were performed in triplicate.

A polynomial quadratic equation for the reducing sugar concentration is given in Eq. (4).

Final equation in terms of coded factors:

Eq (4). Reducing sugar (X) (g/L) = $92.78 + 6.46 * A + 3.01 * B + 12.53 * C - 0.6650 * AB + 1.64 * AC + 0.2425 * BC - 5.71 * A^2 - 6.07 * B^2 + 3.30 * C^2$

Where X is the reducing sugar concentration (g/L), A, B, and C are the coded values of cellulase loading (FPU/g cellulose), hemicellulase loading (U/mL), and hydrolysis time (hour), respectively.

The effect of different parameters on the reducing sugar concentrations of the PR were given in Figure 2. In the response surface graphs, the relationship between the variables and the response was investigated. ANOVA for the model showed that the relationship between variables was high with a good R^2 which was obtained as 0.9529. The lack of fit of the model which is a vital criterion measuring the failure of the model for data representation was also found to be not significant (F value: 0.2797). A non-significant lack of fit is positive for the model and sufficient to estimate response in the presence of various variables (Yücel & Göycıncık 2015). Moreover, according to the model, all the criteria tested were found as significant ($p<0.0001$). Furthermore, hydrolysis time was observed as the most significant parameter ($p<0.0001$) was followed by cellulase loading (p: 0.0002) and hemicellulase loading (p: 0.0214). In Fig. 2a, 35-40 FPU/g cellulose and 35-40 U/mL enzyme loading were observed as sufficient for the sugar released from PR. Reducing sugar amounts did not change dramatically above those enzyme concentrations. This situation depicts the enzyme substrate interaction reached its saturation point (Kim et al. 2008).

On the other hand, hydrolysis time showed the greatest impact on the sugar concentrations, and it was observed that longer hydrolysis time resulted in higher sugar concentrations for both enzymes (Figure 2b and 2c). Similarly, Gul et al. (2018) showed that longer hydrolysis time caused higher saccharification efficiency from Kallar grass. Furthermore, Kshirsagar et al. (2015) found the optimal conditions for reducing sugar yield from rice straw as 40 FPU/g enzyme and 17.50% biomass loading for 72 h when the researchers used RSM for the experiments.

According to the RSM results, the highest reducing sugar concentration was obtained as 108.86 g/L when PR was hydrolyzed with 37.5 FPU/g cellulase, and 37.5 U/mL hemicellulase for 72 h. On the other hand, increased enzyme loading did not cause higher sugar concentrations, and 107.8 g/L reducing sugar was found when the RS was hydrolyzed with 60 FPU/g cellulase, and 60 U/mL hemicellulase for 72 h. Theoretically, increased enzyme loading and extended incubation periods result in higher sugar concentrations. However, the relationship between enzyme loading and sugar concentration may not be linear under all conditions. Loss of the catalytic activity due to the product inhibition, high viscosity and osmolarity or the feedback mechanism may prevent the higher sugar concentrations from increased enzyme loading. Similarly, sugar decreasing trends with the increased enzyme loadings were also reported from sugarcane tops (Sindhu et al., 2014) or *Paspalum scrobiculatum* bran residues (Balakrishnan et al., 2018). The results can also be comparable with ethanol concentrations obtained from sweet sorghum bagasse (Wang et al., 2013) or kitchen wastes (Uncu & Cekmecelioglu, 2011).

Moreover, the maximum ethanol concentration was observed at the end of 72 h. The ethanol concentration of *C. boidinii* increased by 22% and reached to 35.88 g/L under the optimized conditions with RSM in comparison with the experiments carried out without RSM.

Conclusions

It is very important to investigate new raw materials and microorganisms for renewable energy-producing sectors for the sustainability. For these reasons, in the present study, the bioethanol production of newly isolated *C. boidinii* was determined in the fermentation medium which was prepared with PR. Results of the RSM experiments revealed that sugar concentrations of PR increased from 59.80 to 108.86 g/L at the end of 72 h when 37.5 FPU/g cellulose and 37.5 U/mL enzymes were used. Moreover, under these optimized conditions, the highest bioethanol

concentration was observed as 35.88 g/L. This study shows that PR is a promising raw material and *C. boidinii* is an appropriate agent for efficient bioethanol production.

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Author Contributions

First Author: Investigation, writing-original draft; Second Author: Conceptualization, funding acquisition, resources, methodology, writing; Third Author: Review and editing.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper

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Table 1. Effect of increased PR loading on reducing sugar, inhibitory compounds and ethanol concentrations of *C. boidinii* and *S. cerevisiae* (Pre-treatment conditions: 1% H_2SO_4 at 121 °C for 15 min, pH: 5, fermentation time: 48 h)

		Biomass loading (%, w/v)		
		10%	**20%**	**30%**
Reducing sugar (g/L)		43.65±4.67	59.80±4.21	78.32±0.64
Acetic acid (g/L)		0.37±0.07	0.47±0.04	0.81±0.13
Formic acid (g/L)		0.14±0.00	0.20±0.01	0.38±0.06
HMF (g/L)		0.027±0.002	0.063±0.01	0.10±0.03
Ethanol (g/L)	***C. boidinii***	8.55±0.32	21.00±1.31	26.57±3.06
	S. cerevisiae	8.17±0.4	20.67±1.32	24.58±1.00

Table 2. Kinetic parameters of *C. boidinii* and *S. cerevisiae* (Pre-treatment conditions: 1% 447 H_2SO_4 at 121 °C for 15 min, pH: 4.8, cellulase loading: 15 FPU/g cellulose, hemicellulase 448 loading: 15 U/mL, fermentation time: 48 h, initial biomass loading: 20% w/v)

	Initial reducing sugar (g/L)	**Ethanol (g/L)**	**Theoretical ethanol yield (%)**	**Q*p* (g/L.h)**	**Y*P/S* (g/g)**
C. boidinii	81.58±5.44	29.19±0.69	70.0	0.60	0.46
S. cerevisiae		26.92±0.31	64.5	0.56	0.42

Table 3. Types and levels of independent variables and response used in RSM for pre-treatment of pumpkin residues (Initial design: Central composite, Design model: Quadratic)

Response	Factors	Experimental values	
		Lower	**Higher**
Reducing sugar (g/L)	A-Cellulase loading (FPU/g cellulose)	15	60
	B- Hemicellulase loading (u/mL)	15	60
	C- Hydrolysis time (hour)	24	72

Table 4. Experimental responses for reducing sugar concentrations of pumpkin residues using central composite design of RSM (Pre-treatment: 1% H_2SO_4 for 15 min 121 °C, pH: 4.8, initial biomass loading: 20% w/v)

Run No	Factor 1 A: Cellulase FPU/ g cellulose	Factor 2 B: Hemicellulase U/mL	Factor 3 C: Time (hour)	Response 1 Reducing sugar (g/L)	Predicted Reducing sugar (g/L)
1	60	37.5	48	92.50	93.52
2	15	37.5	48	83.25	80.61
3	37.5	37.5	48	93.89	92.78
4	37.5	60	48	92.56	89.72
5	37.5	37.5	48	91.46	92.78
6	37.5	37.5	48	89.53	92.78
7	60	15	24	75.51	74.48
8	15	60	72	91.21	92.64
9	37.5	37.5	48	90.10	92.78
10	37.5	37.5	72	108.86	108.60
11	60	60	24	77.9	78.69
12	37.5	37.5	48	88.19	92.78
13	60	15	72	102.82	102.33
14	37.5	37.5	24	84.91	83.55
15	60	60	72	107.8	107.51
16	37.5	37.5	48	100.24	92.78
17	37.5	15	48	82.48	83.69
18	15	60	24	69.49	70.39
19	15	15	72	85.19	84.80
20	15	15	24	62.82	63.52

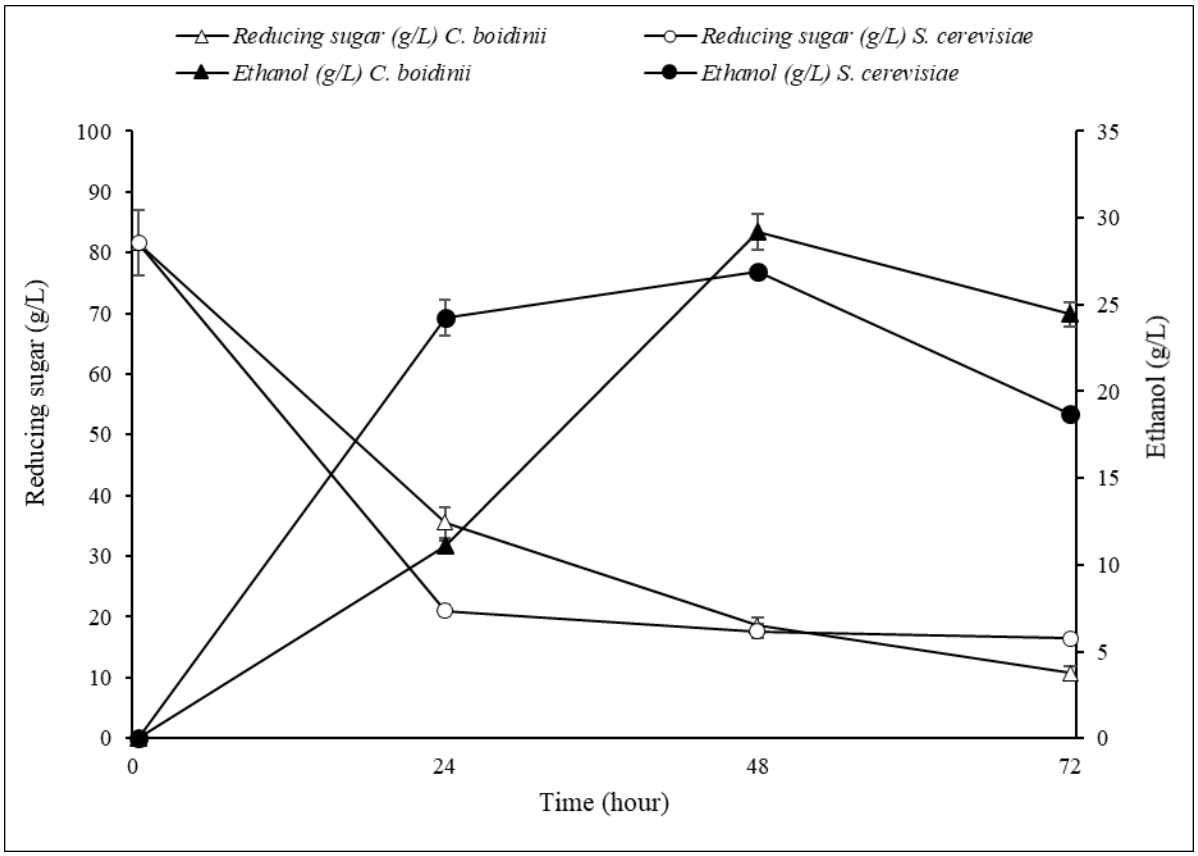

Figure 1. Bioethanol production of *C. boidinii* and *S. cerevisiae* in the presence of dilute acid pre-treated and enzymatically hydrolyzed PR during the fermentation (Pre-treatment conditions: 1% H_2SO_4 at 121 °C for 15 min, pH: 4.8, cellulase loading: 15 FPU/g cellulose, hemicellulase loading: 15 U/mL, initial PR loading: 20% w/v).

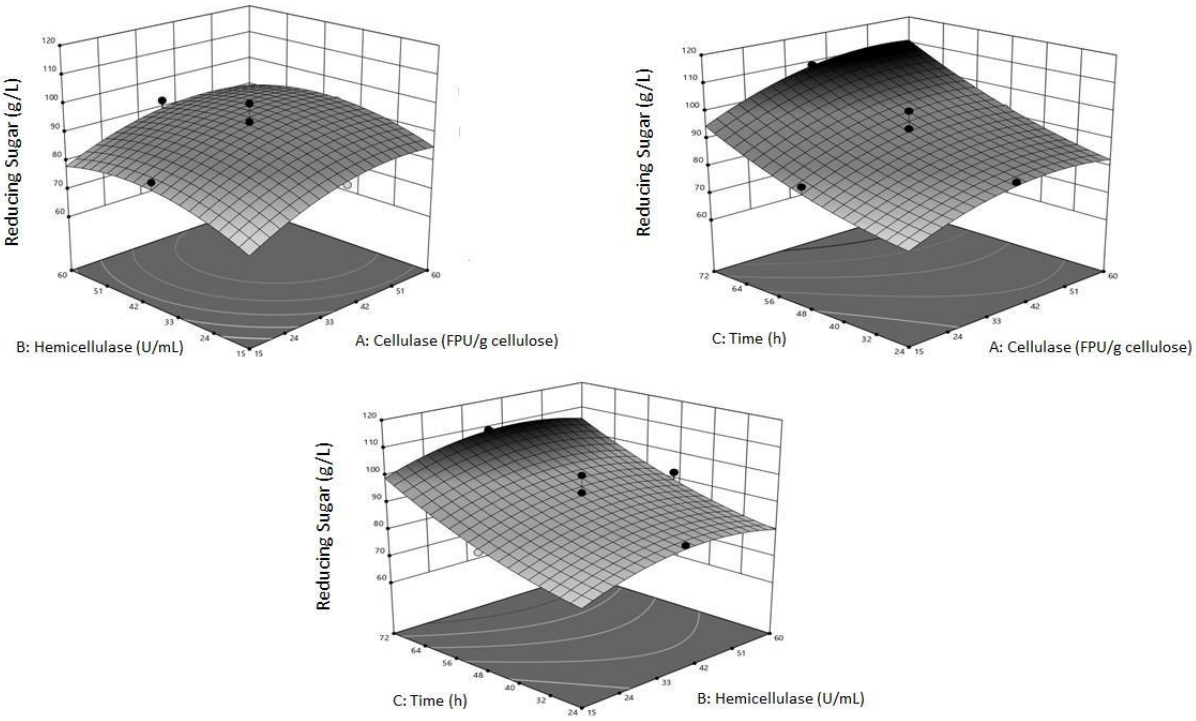

Figure 2. Effect of cellulase, hemicellulase and hydrolysis time on reducing sugar concentrations of PR (initial biomass loading: 20% (w/v), pH: 4.8, Pre-treatment: 1% H_2SO_4 for 15 min 121 °C).

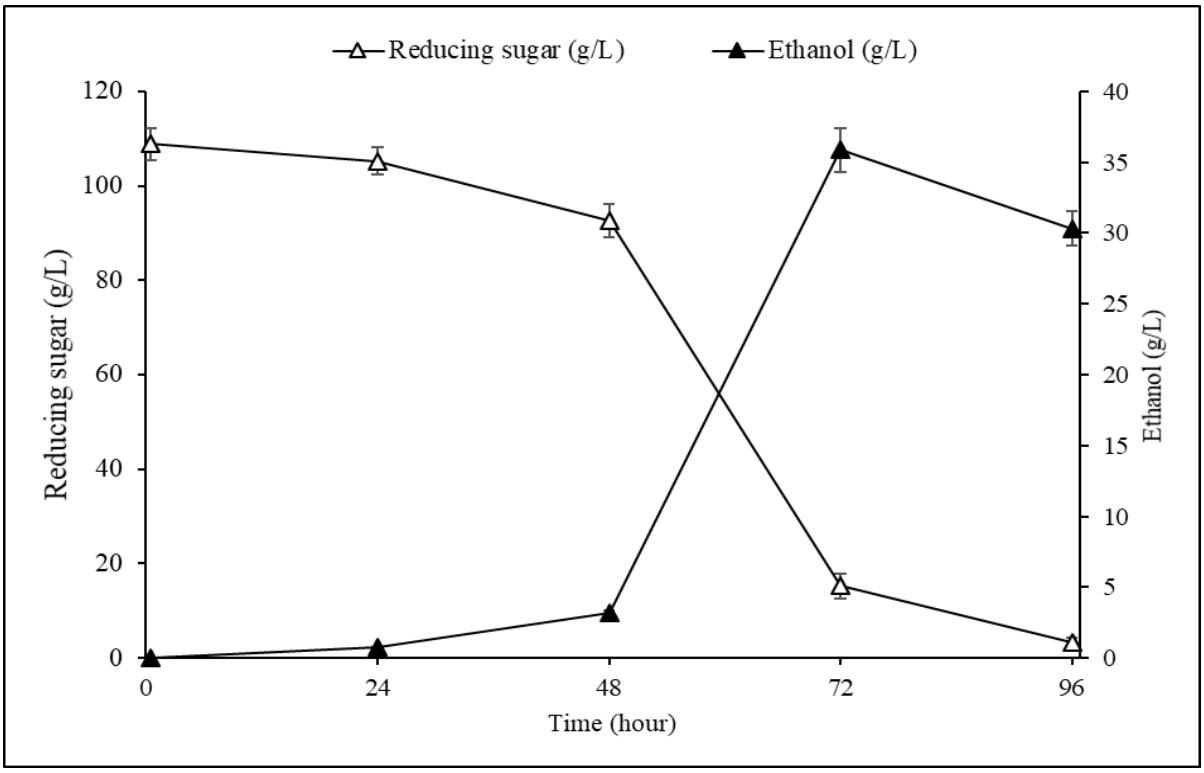

Figure 3. Bioethanol production of *C. boidinii* under optimized conditions (initial biomass loading: 20% (w/v), pH: 4.8, cellulase loading: 37.5 FPU/g cellulose, hemicellulase loading: 37.5 U/mL, enzymatic hydrolysis time: 72 h.