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Simultaneous saccharification and fermentation (SSF) of rice cooker wastewater by using Aspergillus niger and Saccharomyces cerevisiae for ethanol production

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ABSTRACT

This work examined the simultaneous saccharification and fermentation (SSF) process for the biological conversion rice wastewater into ethanol using co-culture of Aspergillus niger (A. niger) and Saccharomyces cerevisiae (S. cerevisiae) in batch condition. In this study, The A. niger and S. cerevisiae were used for hydrolysis and production of ethanol from rice wastewater, respectively. The Effects of fermentation parameters such as pH (4, 4.5, 5 and 5.5), temperature (25, 30, 35 and 40 °C), incubation period (12 to 72 h), incubation time (12 to 72 h) and nitrogen source on SSF were evaluated. The results showed that among the optimal parameters of pH 5, temperature 35 ^{o}C, incubation period 36 h, incubation time 36 h and nitrogen source of $(NH_4)_2SO_4$ were obtained in ethanol production by SSF process. Under these optimized conditions, maximum ethanol production and product yield were 16.97 g/l and 0.36 g/g, respectively.

1. Introduction

One of the most important issues in the 21 century in global communities is the growing demand for energy and providing low-cost and suitable raw materials for production it to be used in different industries, specifically in transportation sectors. In recent years this issue has become evident with an increase in crude oil price. Although, fossil fuel has more than 80 percent of energy consumption in the world's. But in recent decades they have caused many problems for society, including: uneven distribution of the world, Environmental pollution, such as emissions of CO_2, SO_2 and NO_X and Implied Global warming caused by increase greenhouse gases. In today everyone knows that use of fossil fuels led to increasing the global warming. Therefore the most feasible way to meet this growing demand and reduce global warming is by utilizing alternative energies (Najafpour et al. 2004).

Amongst the alternative energies, one of the most important energy sources in near future is biomass. Biofuel is a renewable energy source produced from biomass, which can be used as a substitute for petroleum fuels (Ghorbani et al. 2011).The benefits of biofuels over other fuels such as fossil fuel, include greater energy security, reduced environmental impact, reducing greenhouse gas emissions and Provide Kyoto Protocol, foreign exchange savings and socioeconomic issues. Among the different biofuels, ethanol, due to Inexpensive and appropriate resources, the ability to produce of various resources of sugars, starch and cellulose has attracted the most attention. Ethanol can be used as a biofuel environmentally friendly due to high octane number and existence oxygen in chemical structure. It could be used alone as a fuel or alternative to MTBE in gasoline and to replace MTBE in gasoline. And also it can be used as a carrier of oxygen in gasoline to increase the oxygen content. Ultimately this practice lead to better fuel oxidation and thus reduce the exhaust gas from (Cardona & Sanchez. 2006).

Accordingly, many researchers have investigated the production of ethanol from various sources, such as sugar, starch, cellulose and or amylolytic enzymes. These enzymes could be economically produced by microorganisms (Nigam & Singh. 1995). For example, Ghorbani and coworkers (2011) from cane molasses and Kadar et al. (2004) of industrial wastes. Starchy materials and effluent generated from starch processing units are the abundant, available and inexpensive substrates that can be used as suitable raw materials for ethanol production (Verma et al. 2000). These materials can be easily hydrolysed to fermentable sugars by acid ethanol production from starchy materials such as rice cooker wastewater is a two-step process. The first stage is the saccharification process, which during it the starch converted into simple sugars such as glucose by amylolytic enzymes or acid.

The second stage is the fermentation of sugar derived from hydrolysis in which the sugars derived from saccharification converted to ethanol by microorganisms.

Rice cooker wastewater is one of the most important and-common-effluent urban wastewater. That is produced amount daily and without any use or treatment are disposal into municipal wastewater systems and household. Arriving the effluent to aquatic ecosystems due to high nutrient loads and various actions and anions, causing environmental problems, such as increase COD and BOD ecosystems and creates irreversible damage. The effluent due to high organic matter can be used as an excellent op tion for ethanol production, which is in fact used to be a sustainable biofuel. Since some of the microorganisms used in the fermentation of ethanol, especially, S. cerevisiae and Z. mobilis, are lack of amylolytic enzymes and unable to directly convert the starch into ethanol (Ang et al. 2001; Gupta et al. 2003). Therefore, when using starchy materials as substrates for ethanol production in first must complex structure them broken down into fermentable sugars. There are several technologies available for the conversion of starchy materials to ethanol. The main difference between these technologies are the catalyst and methods used for the brake-down of starch in the fermentable sugars (Kádár et al. 2004). Amongst the different catalyst and methods such as Acid hydrolysis and Separate Fermentation, SSF and Separate hydrolysis and Fermentation, Simultaneous saccharification and fermentation with mixed microorganisms such as

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an amylolytic microorganism and an ethanol-fermenting microorganism is an effective method for the direct fermentation of starch. The advantages of the simultaneous saccharification and fermentation are that a two-stage process for the conversion of starch into ethanol are realized in one reactor and the glucose produced is rapidly converted into ethanol, and the reduced end-product inhibition of the enzymatic hydrolysis, and the reduced investment costs (Beschkov et al. 1984). However, in this system the ethanol yield decreased because much starch was consumed by the growth of amylolytic microorganisms (Nakamura et al. 2000).

The purpose of the present study was to investigate simultaneous saccharification and fermentation (SSF) process of ethanol production from rice cooker wastewater by using Aspergillus niger (A. niger) and Saccharomyces cerevisiae (S. cerevisiae). Also the effect type of nitrogen source on the production of glucose and ethanol in this process by S. cerevisiae and A. niger were evaluated. The glucose production, volumetric ethanol productivity and yield process parameters were examined to describe the consumption of rice cooker wastewater and the production of ethanol.

2. Materials and methods

2.1. Microorganisms

Saccharification and fermentation was performed by A. niger and S. cerevisiae Respectively (Persian Type Culture Collection, PTCC 5010) supplied from the Research and Technology of Ministry of Sciences (Iran) in the form of freeze-dried culture in the form of freeze-dried culture.

2.2. Media

The medium for the A. niger cultivations were as follows: It was cultured in a sterilized liquid medium, propagated in nutrient agar and then stored in a refrigerator at 48 °C. The composition of the growth medium was (in g/l): sucrose, 50; NH_4NO_3, 2;KH_2PO_4, 0.15; $MgSO_4$, 0.15; $FeSO_4.7H_2O$, 0.005; $MnSO_4.H_2O$, 0.016; $CoCl_2.6H_2O$, 2.9; $ZnSO_4.7H_2O$, 0.0014. The medium was sterilized by autoclaving at a pressure of 1atm and a temperature of 1218 °C for 20 min. The temperature and the pH of the growth medium were at ambient temperature 30 °C and pH 5.5, without shaking. The fungal cells were grown for 5 days (end of the exponential phase) and then filtered (0.451 m pore size). The medium for S. cerevisiae cultivations were as follows: The culture was maintained on a sterilized solid Potato Dextrose Agar (PDA) medium in a 20 ml-test tubes and transferred to fresh medium every six months. The culture was incubated at 30°C for 1 - 3 days and stored at 4 °C until use. Before starting the experiment, the microorganism was inoculated under sterile conditions into glass test tubes containing the same solid culture medium. These tubes were then kept in an incubator at 30 °C for 16 h in order to obtain cells at the same growth stage for every experiment. The composition of the media was (in g/l): glucose, 15; $(NH_4)_2SO_4$, 9; $MgSO_4.7H_2O$, 2.5; yeast extract, 1; KH_2PO_4, 10; K_2HPO_4, 5. The media were sterilized in an autoclave (Reyhan Teb, F2000, Iran) at 121 °C and 1 atm for 20 min. The pH was maintained at 4.5 by the addition of either 1 N NaOH or 1 N HCl when necessary.

2.3. Characteristic of the rice cooker wastewater

Rice cooker waste water is one of the most abundant urban and household wastewater effluents that is produced from different places such as restaurants, hotels and houses. The supply of rice cooker wastewater as municipal effluent, used in this study and provided by restaurant's university of Natural Resources and Marine Sciences, Tarbiat Modares University (Noor, Iran).

2.4. Simultaneous Saccharification and Fermentation (SSF)

Saccharification and fermentation of rice cooker wastewater were performance simultaneously in the batch culture at temperature 30 °C and pH 5 by using the A. niger and S. cerevisiae for hydrolysis fermentation. For this experiment prior to SSF, in first rice cooker wastewater was concentrated to the glucose that it arrived to the 50 g/l. The SSF experiments were performed in 500 ml E-flasks. Each flask contained 200 ml of culture medium in which the concentrations of nutrients were (in g/l): $(NH_4)_2SO_4$, 9; $MgSO_4.7H_2O$, 2.5; yeast extract, 2; KH_2PO_4, 10; K_2HPO_4, 5; $FeSO_4.7H_2O$, 0.005; $MnSO_4.H_2O$, 0.016; $CoCl_2.6H_2O$, 2.9; $ZnSO_4.7H_2O$, 0.0014. The A. niger fungal was inoculated on fermentation medium. The sampling time for reducing sugar concentration analysis was 12, 24, 48 and 72 h and then the medium was incubated with three present S. cerevisiae in different times (12, 24, 36, 48 and 60 h) to the fermentation medium and the sampling time for ethanol production analysis was every 12 h, respectively. The flasks were incubated in a rotary shaker at 30 °C for 72 h samples were withdrawn regularly every 12 h, centrifuged in a laboratory desktop centrifuged for 15 min at 6000 × g, and the supernatants were analyzed for determination glucose and ethanol. All experiments were performed in triplicate and the average values are presented.

2.4.1. Effect of pH and temperature on ethanol production

Effect of pH and temperature on ethanol fermentation by the process of simultaneous saccharification and fermentation using mixed cultures of S. cerevisiae and A. niger was carried out by varying the pH, (4, 4.5, 5 and 5.5) and temperatures (25, 30, 32 and 35 ºC).

2.5. Effect of incubation time on glucose and ethanol production

For determining the effect of incubation time on glucose and ethanol production, 200 ml of rice cooker wastewater substrate was dispersed to 500 ml E-flasks. In first step the A. niger was incubated in the medium and every 12 h samples were taken to determine the amount of glucose produced by A. niger. And in second step the S. cerevisiae was incubated in different times (12, 24, 36, 48, 60 and 72 h) every 12 h samples were taken to determine the amount of glucose consumption and ethanol production.

2.6. Effect of nitrogen source on glucose and ethanol production

The effects of nitrogen source ($(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl) were investigated on the hydrolysis of rice cooker wastewater and ethanol production by A. niger and S. cerevisiae at a pH and temperatures were adjusted to 5 and 30 °C, respectively. The flasks were incubated in an orbital shaker with a speed of 120 rpm. The reaction time was set 72 h. Samples were periodically withdrawn up to 12 h to monitor the extent of glucose and ethanol produced by A. niger and S. cerevisiae, respectively. Under this condition, the S. cerevisiae was incubated into medium after 36 h as A. niger.

2.7. Analytical methods

In this study, samples for analysis of glucose and ethanol contents were first centrifuged for 15 min at 6000 × g and the supernatants were analyzed for determination glucose and ethanol. Reducing sugar concentration was estimated by DNS method (Miller. 1959). Ethanol concentration was determined by gas chromatography (Philips, PU440, US) using flame ionization detector and with software (Clarity 4.2, Data Apex Czech Republic) used to analyze the liquid samples. The column used was PEG 20 M (glass column) 1.5 m and 1/8 mm (Philips, USA). Temperature programming was employed for the liquid analysis in GC. During the analysis, the column temperature was initially maintained at 120 °C and after 2 min the oven temperature was increased at a rate of 10 °C/min until it reached to 150 °C. The injector and detector temperatures were maintained at 220 °C. Nitrogen was used as the carrier gas at 30 ml/min. Acetone (1 %, v/v) was used as an internal standard with concentration of 20 ml/ml per sample. The injection sample volume was 2 µl. Each set of the experiment and the data points were repeated three times. The reported value was the average.

3. Results and discussion

Starchy materials and effluent generated from the starch generating unit such as rice mil are the cheap and abundant substrates that could be used as potential raw materials for ethanol fermentation. But these materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). In the present study the ethanol production of rice cooker wastewater, as a Starchy material, was investigated in SSF experiments. By using the A. niger and S. cerevisiae for saccharification and fermentation respectively at a pH and temperatures were adjusted to 5 and 30 °C, respectively. The flasks were incubated in an orbital shaker with a speed of 120 rpm. The reaction time was set 72 h.

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3.1. Effect of pH and temperature on ethanol production

Fig.1. shows the result of the effect of pH and temperature on ethanol concentration. Effects of temperature to determine the optimum temperature, experiments were conducted at different temperatures (25 to 40 °C). It was observed that the ethanol production increased (11 g/l) by increase in temperature up to 30 °C (Fig. 1) for rice cooker wastewater, but above this the productivity decreased though there was an increase in SSF of the rice cooker wastewater. However, temperatures beyond 30 °C showed a fall in ethanol production which is in line with the findings of Sharma et al. (2007), who reported optimum temperature for simultaneous saccharification and fermentation of kinnow waste and banana peels was found to be 30 °C with maximum ethanol yield of 0.376 g/g and fermentation efficiency of 74.11 % (Sharma et al. 2007). Verma et al. (2000) also reported 30 °C as the optimum temperature for maximum ethanol production using starch employing co-culture of amylolytic yeast and S. cerevisiae (Verma et al. 2000). Among the physical parameters, the pH of growth medium has played an important role by inducing morphological change in the organism and in enzyme secretion. This result shows the optimum pH range of 5 gave the optimum yield of glucose. This corroborates the results of Aderemi et al. (2008) at which the optimum glucose yields were obtained range between at pH 4.5 and 5. The productivity decreased by decrease or increase in pH of the medium. This may be due to the low activity of enzyme that is involved in the process.

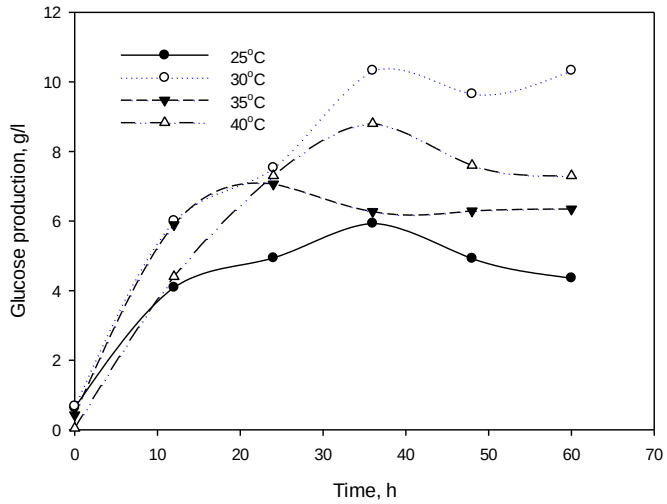

Fig.1. Effect of pH on co-culture A. niger and S. cerevisiae cell activity on rice cooker wastewater for ethanol production.

The results of the effect of different pH on the ability of the fungus in hydrolysis of effluent showed that pH 5 have greatest effect on glucose production by A. niger in temperature 35 °C. The results of this experiments according to Sohail et al. (2009) that they studied the effect of pH and different temperatures on growth of A. niger of the cellulose producing. Although most fungi are active in the range of pH 4 to 6, but it is necessary to increase the efficiency of their operations, the appropriate pH was optimized. Because changing in pH causes changes in protein composition of the cell wall. For example, reducing pH causes the membrane fatty acids to become more saturated forms. Thus, this is getting stronger and impenetrable thereby reducing microbial growth and activities brings. Pedersen et al. (2000) have studies the effect of pH 2.5 to 6 on the growth and production of starch degrading enzymes. Their results showed that changes pH does not have much impact on growth and ability to produce enzymes glucoamylase. However, their results showed that the maximum amount of enzymes produced was at pH 4 to 5.5, And that the highest enzyme production was observed at pH 5.

3.2. Effect of incubation time on glucose and ethanol production

In SSF process, the incubation time is the limiting factor for fungus and yeast growth or ethanol production. Therefore, the effect of incubation time on Saccharification and fermentation using co-culture A. niger and S. cerevisiae was examined at 0 to 72 h, at a pH and temperatures to 5 and 30 °C, respectively. Fig. 2 shows the reducing sugar and ethanol concentration produced from enzymatic saccharification and fermentation by A. niger and S. cerevisiae. It was found that the reducing sugar concentrations increased with increase in time up to 48 h but above this the productivity decreased. The results show that the maximum reducing sugar concentrations were 27.2 g/l in during 48 h after incubation of A. niger though there was an increase in saccharification of the rice cooker wastewater. This can be caused by breaking starch and glucose production piece, and then consumption piece is by yeast. Results revealed that A. niger can be utilized 53 percent of 50 grams per liter of total sugar. In fact, this show has the ability to hydrolysis of rice cooker wastewater by A. niger. And glucose produces for ethanol production by S. cerevisiae. The result of incubation time S. cerevisiae for ethanol production showed when the incubation time were varied (12 to 72 h) these results were different (Table 1). The effect incubation time of S. cerevisiae on ethanol production shows that the maximum ethanol produces in 36 h after incubation of A. niger 11.26 g/l. (Fig. 2).There might be an increase in saccharification over the period making glucose available to S. cerevisiae for fermentation. Olofsson et al. (2008) reported that enzymatic hydrolysis of the solid fraction has a large control over the total rate of ethanol production in SSF. In a similar study carried out on effect of incubation period on ethanol productivity, Sharma et al. (2007), has reported maximum ethanol yield and fermentation efficiency of 0.397 g/g and 77.84 percent, respectively after 36 h of incubation at 30 °C using mixed culture of S. cerevisiae and P. tannophilus. Reported the maximum ethanol production (0.398 g/g) of at 48 h incubation of employing process of Co-culture of S. cerevisiae G and P. tannophilus MTCC 1077 along with enzymes exhibited. Some authors have reported maximum glucose yield after 48 h of incubation from starchy materials.

3.3. Effect nitrogen source on glucose and ethanol production

Nitrogen is one of the main elements found in many macromolecules of living organisms, playing a central role in structure and function (Magananik. 2005; Najafpour et al. 2004). Type and concentration of nitrogen source effect on fungal growth because it is not only important for metabolic rates in the cells but it is also the basic part of cell protein. In this study the effect of nitrogen source on glucose and ethanol production by A and S were investigated. $(NH_4)_2SO_4$ give the best results, producing 24.63 g/l glucose directly from rice cooker wastewater after 48 h. Also results show NH_4Cl has lowest effect on glucose production.

Table 1. Effect of incubation period and incubation time on ethanol production in SSF process.

Time, h	Incubation time	Incubation period
	Ethanol concentration, g/l	Ethanol concentration, g/l
12	3.56	3.86
24	6.7	5.9
36	12.34	13.6
48	11.26	12.2
60	10.87	11.98
72	10.16	12.34

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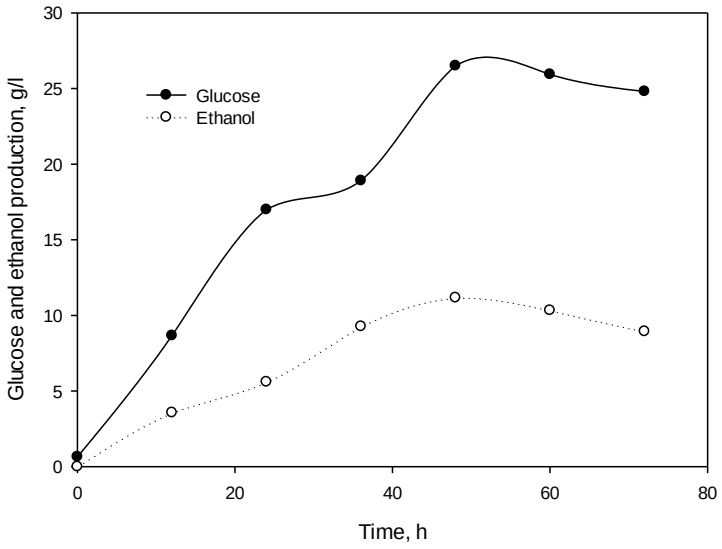

Fig. 2. Effect of incubations time on glucose and ethanol production.

According to Acourene of Ammouche (2010), the nature and relative concentration of nitrogen source are important in formation of α-amylase. Among the organic sources, the yeast extract is the best nitrogen source that increased in the amylase activity. They express that among inorganic nitrogen source ammonium nitrate also enhanced α-amylase activity relatively and but ammonium chloride, repressed the enzymes production.

Nitrogen and complexity of the nitrogen source, strongly affect the glucose fermentation. Effect of nitrogen source was investigated on ethanol production in (SSF) co-culture A. niger and S. cerevisiae the result show that NH_4NO_3 have the highest effect on ethanol production in co-culture A, and S. with 16.97 g/l. Also ammonium sulfate Compared to NH_4NO_3 has lower effect on ethanol production. According Júnior et al. (2008) ammonium sulfate always induced poorer fermentation performance, with lower biomass and ethanol production, and loss of yeast viability. Their result is similar to that of this study. Among the nitrogen source NH_4Cl have the lower effect on ethanol production. In general, during the SSF experiment with both A. niger and S. cerevisiae, the rate of hydrolysis was lower than the rate of glucose consumption by the yeast cell, which resulted in glucose complete consumption in the fermentation broth.

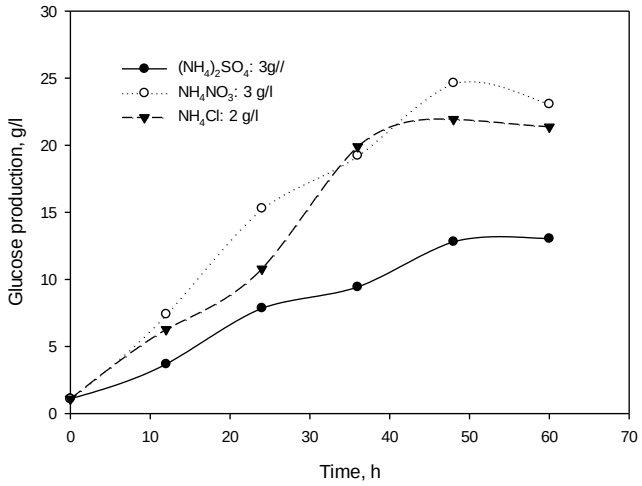

Fig.3. Effect of N course on glucose production by A. niger.

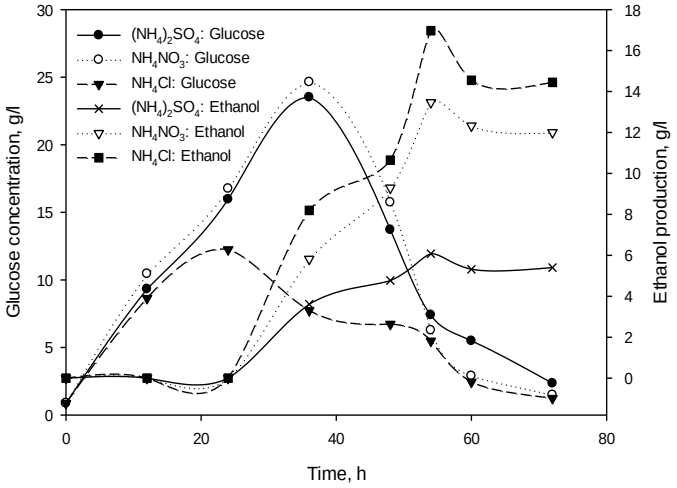

Fig. 4. Effect of nitrogen source on glucose and ethanol production by A. niger and S. cerevisiae in SSF process.

The result show that the between type nitrogen source and maximum glucose and ethanol production sole A. niger and co-culture A. niger and S. cerevisiae have different Fig.4. This may be due to affected structural complexity of the nitrogen source on fungus and yeast metabolism (Ghorbani & Younesi. 2013; Ghorbani et al. 2011). According Messias et al. (2008) and Cruz et al. (2002) have shown that the structural complexity of the nitrogen source strongly affected yeast metabolism.

4. Conclusion

This work thus demonstrates the simultaneous saccharification and fermentation (SSF) process for the biological conversion rice wastewater into ethanol using co-culture of A. niger and S. cerevisiae in batch condition. The process fairly described glucose liberation from SSF and biomass growth, total sugar consumption, ethanol formation, accumulation of ethanol inhibition from fermentation in a batch experiments. The results of this study indicated that the maximum substrate consumption rate was inhibited by formation of ethanol in batch condition. The results showed that highest glucose production observed using $(NH_4)_2SO_4$ as nitrogen source in SSF process. From an engineering point of view, these alternatives exhibited comparable biological activity in comparison to the ethanol obtained using the more costly feedstocks and has the potential to become an environmentally and economically acceptable technology for biofuel production.

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