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Simultaneous Saccharification and Fermentation of Corn Husk by Co-Culture Strategy

Shivani Sharma, Vinay Sharma and Arindam Kuila*

Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India

Abstract

Lignocellulosic biofuel production mainly carried out by two ways: simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). In the present study, simultaneous saccharification and fermentation (SSF) was carried out using microwave assisted thermochemically pretreated (0.5 M NaOH for 20 minutes at 120°C in preheated oven) corn husk. Using co-cultures of *Saccharomyces cerevisiae* and *Fusarium oxysporum*, SSF process was optimized. Maximum ethanol production (6.24%, v/v) was observed after 24 h of incubation. Further for enhanced ethanol production, effect of different surfactant was carried out on SSF using co-culture strategy. It was found that addition of Tween 60 enhanced the ethanol production upto 6.38% (v/v). Further for addition enhancement of ethanol production, different co-culture strategy was adopted. It was found that maximum ethanol production (6.58% v/v) was obtained when ethanol fermentation was carried out by *Fusarium oxysporum* followed by *Saccharomyces cerevisiae*.

Keywords: SSF; Microwave assisted thermochemical pretreatment; Corn husk; Co-culture strategy; Tween 60

Introduction

The use of bioethanol as fuel is increasing day by day as it is one of the most sustainable sources of energy which causes no harm to the environment [1]. Now-a-days bioethanol production from lignocellulosic biomass is gaining great impetus because of its blending with gasoline which increases the octane level, consequently diminishing the CO_2 emissions by 12-15% over gasoline. Ethanol yields 25% more energy compared to total energy required for cultivation of lignocellulosic biomass and its use for ethanol production [2]. Lignocellulosic biomass is mostly preferred for bioethanol production as it is available in huge amounts all over the world [3]. Bioethanol is produced from the lignocellulosic biomass by following certain steps like: pre-treatment of lignocellulosic biomass, saccharification/enzymatic hydrolysis, fermentation and distillation [4-6].

Pretreatment is important as it enhances the accessibility of the substrate for efficient hydrolysis and biofuel production. Several methods have been developed for the pretreatment of lignocellulosic biomass such as mechanical, thermal, chemical and biological and their combinations thereof [7,8]. After pretreatment saccharification is done in which the biomass polymers/carbohydrates are converted into fermentable sugars. Saccharification of lignocellulosic biomass is mainly carried out by cellulase enzyme due to its high specificity and higher sugar yield [9]. Cellulases used for saccharification can be obtained from several species of algae, fungi and bacteria. Fermentation is carried out after saccharification by a variety of microorganisms such as fungi, bacteria, and yeasts. *Saccharomyces cerevisiae* is one of the widely studied and used yeasts for industrial ethanol production because of its ability to produce high concentrations of ethanol as well as its high tolerance to ethanol and other inhibitory compounds [10]. To enhance the process of bioethanol production the carbohydrates present in the lignocellulosic biomass can be converted into ethanol by Seperate hydrolysis and fermentation (SHF), Simultaneous saccharification and fermentation (SSF) or Simultaneous saccahrification and cofermentation (SSCF). SSF is preferred over other as in this the sugar produced by enzymatic hydrolysis is simultaneously fermented by microorganisms like *Saccharomyces cerevisiae* and this whole process of simulation saccharification and fermentation can take place in a single reactor reducing the chances of contamination or product inhibition of enzymes [11]. The other approach is to utilize two microorganisms (*Zymomonas mobilis* and *Acetobacter sp.*) at the same time, which is called "co-culture" (two microorganisms are cultured together and simultaneously exist in the same medium). Utilization of co-cultures for ethanol production appears to have advantages over single culture since there is potential for synergistic action of the metabolic pathways of all involved strains [12]. *Saccharomyces cerevisiae*, which is by far the dominant yeast used for ethanol production, naturally converts glucose to ethanol but does not metabolize xylose [10,13]. Co culturing the *Saccharomyces cerevisiae* that prefer six-carbon sugars with yeasts that produce efficient ethanol from five-carbon sugars is also another alternative to optimize ethanol in hydrolysates containing xylose [14,15]. Coculture of *Saccharomyces cerevisiae* ITV-01 and *Pichia stipitis* NRRL Y-7124 showed fivefold increase in ethanol productivity compared to monocultures [14]. The present study was focused on simultaneous saccharification and fermentation (SSF) of thermochemically pretreated corn husk using *Fusarium oxysporum* and *Saccharomyces cerevisiae*. SSF process was optimized by statistical technique. Effect of supplementation of different non-ionic surfactant was studied for further enhancement of ethanol production. In addition, different co-culture strategies were also adopted for additional increased ethanol production.

Materials and Methods

Biomass

Corn husk was collected from nearest mill of Banasthali, Rajasthan, India. It was oven dried at 70°C and further grinded to particle size

***Corresponding author:** Arindam Kuila, Department of Bioscience and Biotechnology, Banasthali University, Rajasthan-304022, India, Tel: +91-1438-228302, Fax: +91-1438-228365; E-mail: arindammcb@gmail.com

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less than 0.2 mm. Biochemical constituents (lignin, cellulose and hemicelluloses) were quantified using standard protocols [16].

Microwave assisted thermo chemical pretreatment

The milled corn husk (8%, w/v) was treated with 0.5 M NaOH for 20 minutes at 120°C in domestic micro oven (Whirlpool Household Microwave Oven, Magicook 20S (M). After pretreatment, biomass was washed with tap water and dried at 70°C for further use.

Cellulase production

Cellulase production was carried out using locally isolated *Fusarium incarnatum* KU377454 under submerged fermentation in 500 mL Erlenmeyer flask which contained 100 mL of sterile minimal medium and milled groundnut shell (8 g/100 mL of medium). The culture medium was incubated at 28°C for 3 days. After incubation, the culture medium was filtered using a muslin cloth and the crude enzyme extract thus obtained, was centrifuges at 5000 rpm for 5 min and clear supernatant was separated. The FPase activity of the supernatant was measured by following the procedure of Nathan et al. [17]. The clear supernatant was used as crude enzyme which was stored in capped glass bottles at 4°C for further experiments.

Simultaneous saccharification and fermentation (SSF)

Pretreated corn husk was added with required volume of crude cellulase enzyme (20 IU/gds) and kept at 50°C for 48 h. The pH of the broth was measured as 5. After 48 h of hydrolysis, samples were taken and centrifuged at 5,000 rpm for 10 min. Further, supernatant was collected, and the reducing sugar was measured by DNS method [18]. Baker's yeast, *Saccharomyces cerevisiae* was used in the present study for bioethanol production from hexose sugars. Locally isolated *Fusarium oxysporum* was used for ethanol production from pentose sugars. Pretreated corn husk was inoculated with co-culture of both the yeast strains in different ratio along with required volume of crude cellulase enzyme. Yeast extract (2 g/L) was added as an additive to the fermentation medium for enhanced growth. The flasks were covered with parafilm in order to provide semi-anaerobic conditions for the yeast and incubated at 37°C . After that ethanol production (%, v/v) was quantified by HPLC method [19].

Optimization of simultaneous saccharification and fermentation of thermochemically pretreated biomass

In the present experiment, central composite design (CCD) based on response surface methodology (RSM) was used to examine the effects of several environmental parameters on simultaneous saccharification and fermentation of pretreated substrate [20]. The design matrix with 20 experimental runs with five replicates of the midpoint has been shown in Table 1. The ranges of each parameter were substrate concentration (18-22%, w/v), temperature (30-40°C) and incubation time (24-48 h). The experimental data were analyzed by the Response Surface Regression (RSREG) procedure to fit the following second-order polynomial equation:

$$Y = \beta_{k0} + \Sigma_{i=1}^{5} \beta_{ki} x_i + \Sigma_{i=1}^{5} \beta_{kii} x_i^2 + \Sigma_{i=1}^{4} \Sigma_{j=i+1}^{5} \beta k_{ij} x_i x_j \quad (1)$$

Where Y was response ethanol production (mg/mL); β_{k0}, β_{ki}, β_{kii} and βk_{ij} were constant coefficients and x_i, x_j were the coded independent variables, which influence the response variables Y. This response was preferred because a relatively few experimental combinations of the variables were adequate to estimate potentially complex response function. Data were analyzed using Minitab 15 programs to find out the interaction between the variables and the responses.

Effect of non-ionic surfactant on SSF of pretreated corn husk

SSF of pretreated corn husk was carried out at optimized conditions and was supplemented with various non-ionic surfactants (Tween 20, Tween 40, Tween 60, Tween 80) at constant concentration (0.1%).

Effect of different ratio of *Fusarium oxysporum* and *Saccharomyces cerevisiae* on SSF of pretreated biomass

In order to study the effect of different way of co-culture strategy, ethanol production was carried out at optimized conditions (substrate concentration 22% (w/v), temperature 30°C , Tween 60 concentration 1% and incubation time 24 h) by varying ratio of co-culture of *Fusarium oxysporum* and *Saccharomyces cerevisiae*.

Results and Discussion

Biochemical constituents of corn husk

Table 1 showed major biochemical constituents of corn husk. It showed that pretreatment didn't alter the content of cellulose, but lignin and hemicelluloses contents were drastically reduced. Cellulose content of untreated substrate was nearly equal to the previous report [21]. But, lignin and hemicellulose percentages in the substrate were found to be different than earlier report [21]. Lignin present in the substrate act as a hindrance for enzymatic hydrolysis. Therefore, in this study biomass was thermo chemically pretreated before enzymatic hydrolysis. Biochemical characterization confirmed the candidature of thermo-chemical pretreated sample as a perfect substrate to be used for cellulase production.

Optimization of simultaneous saccharification and fermentation by co-culture strategy

Simultaneous saccharification and fermentation process of pretreated substrate was optimized using CCD based RSM. Table 2 showed the experimental design and response for simultaneous saccharification and fermentation of pretreated substrate. Interactive effect of the independent variables (substrate concentration, temperature and incubation time) was investigated to obtain optimum conditions of ethanol production. ANOVA analysis (Table 3) carried out that gave following second order polynomial model:

Ethanol (%, v/v) = + 95.87 – 5.76 × substrate concentration – 1.04 × temperature – 0.78 × incubation time + 0.11 × substrate concentration × substrate concentration + 0.012 × substrate concentration × temperature + 0.03 × substrate concentration × incubation time (2).

From ANOVA table it was found that the F value was 2593.96 and P value was <0.001 at 9 degree of freedom. The obtained F value was lesser than table F value and consequent P value was very less (less than 0.05), which showed that the RSM model adequately describe the relationship between the response (ethanol production) and the independent variables. This demonstrated that the present model was

Lignocellulosic biomass type	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Solid yield (%)
Control	38.50	25.50	23.40	100.00
Microwave assisted dilute alkaline pre-treated substrate	49.05	11.35	7.55	65.50

Table 1: Biochemical composition of corn husk.

Run order	Substrate concentration (%)	Temperature (°C)	Incubation time (h)	Ethanol (%, v/v)	
				Experimental	Predicted
1	18	30	24	6.28	6.27
2	22	30	24	4.32	4.33
3	18	40	24	4.55	4.56
4	22	40	24	3.10	3.09
5	18	30	48	3.75	3.76
6	22	30	48	4.57	4.57
7	18	40	48	3.75	3.74
8	22	40	48	5.01	5.02
9	18	35	36	4.52	4.52
10	22	35	36	4.20	4.19
11	20	30	36	4.42	4.42
12	20	40	36	3.80	3.79
13	20	35	24	3.96	3.96
14	20	35	48	3.69	3.68
15	20	35	36	3.95	3.93
16	20	35	36	3.90	3.93
17	20	35	36	3.92	3.93
18	20	35	36	3.91	3.93
19	20	35	36	3.94	3.93
20	20	35	36	3.95	3.93

Table 2: Experimental design and responses for simultaneous saccharification and fermentation of pretreated substrate.

Source	DF[a]	Seq SS[b]	Adj SS[b]	Adj MS[c]	F	P
Regression	9	7.9285	7.9285	0.88094	2593.96	<0.001
Linear	3	1.4593	5.94821	1.98274	5838.21	<0.001
Square	3	1.15236	1.15236	0.38412	1131.05	<0.001
Interaction	3	5.31684	5.31684	1.77228	5218.52	<0.001
Residual Error	10	0.0034	0.0034	0.00034	--	--
Lack-of-Fit	5	0.00111	0.00111	0.00022	0.49	0.775
Pure Error	5	0.00228	0.00228	0.00046	--	--
Total	19	7.9319	--	--	--	--
R^2	99.96%	99.92%	--	--	--	--

[a]Degree of freedom, [b]Sum of squares, [c]Mean squares

Table 3: ANOVA of RSM model for simultaneous saccharification and fermentation of pre-treated biomass.

capable of describing maximum variation in the data. The interactive effect of independent variables was observed using 3D response surface plot analysis (Figures 1-3). Each figure represents the effect of two different independent variables on ethanol production while other parameters kept constant at its optimum point. From 3D response surface plot analysis, the optimum predicted conditions for ethanol production was: substrate concentration 22% (w/v), temperature 30°C and incubation time 24 h. Under above conditions maximum experimental ethanol production was found to be 6.24% (v/v), which was very close to predicted response (6.27%, v/v). Bhatia and Johri reported that response analysis revealed the maximum ethanol concentration (10.1986 g/L) by *Pachysolen tannophilus* could be achieved at the optimum process conditions from bagasse [22]. Co-culture bioconversion is a very plausible and potentially high-payoff opportunity for ethanol production. The idea of using a co-culture approach for production of ethanol is to combine a xylose-fermenting microorganism and a glucose-fermenting microorganism to ferment glucose and xylose simultaneously. Sangharak reported maximum bioethanol production (2.66%, v/v) after 36 h of SSF of pretreated office paper [23]. It is possible to obtain relatively high bioethanol production efficiency by using optimized fermentation parameters. An overall economic process must include achieving a high bioethanol yield (5-8%, w/v) at high substrate loading (>10%, w/v) over short residence times (<5 days), most of which were achieved in the present study [24].

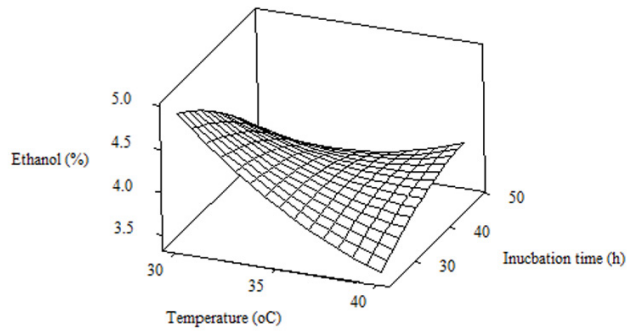

Figure 1: Effect of temperature and incubation time on simultaneous saccharification and fermentation of pre-treated substrate.

Effect of various non-ionic surfactants on SSF using co-culture

In the present study, effect of different non-ionic surfactants was studied on SSF of pretreated biomass. It was observed that maximum

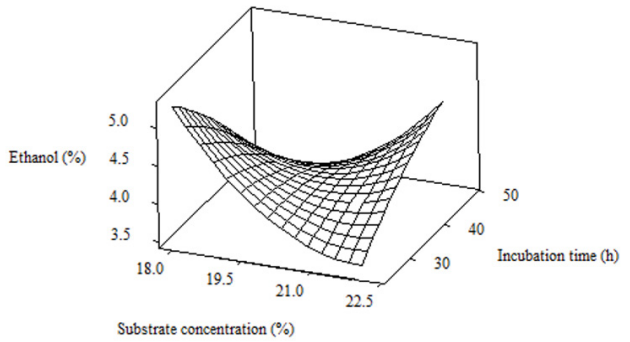

Figure 2: Effect of substrate concentration and incubation time on simultaneous saccharification and fermentation of pre-treated substrate.

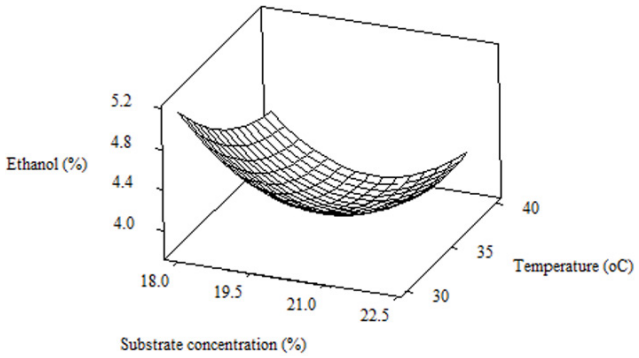

Figure 3: Effect of substrate concentration and temperature on simultaneous saccharification and fermentation of pre-treated substrate.

ethanol yield (6.38%, v/v) was obtained in presence of Tween 60 (Figure 4). Liu et al. observed that the surfactants Tween 20, Tween 80, and BSA increased glucan and xylan conversion, and hence increased ethanol concentration and ethanol yield, compared with no addition of surfactants [25]. McIntosh et al. [26] reported addition of polyethylene glycol increased the ethanol titers and yields upto 60 g/L and 95%, respectively at high solid (20%, w/v) simultaneous saccharification and fermentation of pretreated biomass.

Different strategy of using *Fusarium oxysporum* and *Saccharomyces cerevisiae* for ethanol fermentation

In order to study the effect of different ratio of *Fusarium oxysporum* and *Saccharomyces cerevisiae* on SSF of pretreated substrate, ethanol production was carried out by using their different proportions (Figure 5). It was observed that the maximum ethanol production (6.38%, v/v) was obtained when sugar fermentation was carried out at equal ratio of *Fusarium oxysporum* and *Saccharomyces cerevisiae*. In another strategy, under previous optimized conditions, 5% of *Fusarium oxysporum* was inoculated and incubated for 24 hours at 30°C. After that *Fusarium oxysporum* was inactivated by heat treatment (80°C for 30 minutes). Then reaction mixture was cooled to room temperature and further inoculated it with 5% of *Saccharomyces cerevisiae* and incubated for 24 hours at 37°C again. This condition of co-culture of *Fusarium oxysporum* and *Saccharomyces cerevisiae* provided the maximum bioethanol production (6.58%, v/v). In earlier report, Neves et al. [27] reported a combination of *Candida shehatae* and *Saccharomyces cerevisiae* to be an effective combination for maximum ethanol production from pretreated substrate [27]. They reported that both of the microbes (*Candida shehatae* and *Saccharomyces cerevisiae*) were compatible to operating pH and temperature. Co-culture of *Saccharomyces cerevisiae* ITV-01 and *Pichia stipitis* NRRL Y-7124 was investigated by Gutierrez-Rivera et al. [14]; they found that ethanol productivity increased fivefold compared to monocultures. They reported maximum ethanol production (4.42%, v/v) using co-culture of *Saccharomyces cerevisiae* ITV-01 and *Pichia stipitis* NRRL Y-7124. However, the problem in this coculture was that *P. stipitis* NRRL Y-7124 tolerated lower ethanol inhibition than *Saccharomyces cerevisiae* ITV-01 and hence the ethanol concentration produced by *Saccharomyces cerevisiae* ITV-01 prevented further ethanol production in *P. stipitis* NRRL Y-7124 [14]. Similarly, the co-culturing of *Saccharomyces cerevisiae* MTCC 174 and *Scheffersomyces stipitis* NCIM No. 3497 (formerly *P. stipitis*) was studied using microwave alkali pretreated rice husk medium; it was reported that their co-culture produces maximum ethanol concentration (20.8 g/L) compared to *Saccharomyces cerevisiae* MTCC 174 (14.0 g/L) and *S. stipitis* NCIM No. 3497 (12.2 g/L) alone [15]. Harish et al. [28] recently showed that co-culture fermentation of *C. thermocellum* with *C. thermosaccharolyticum* on banana argowaste hydrolysate with maximum ethanol of 0.41 g/g was more efficient in terms of ethanol production, cellulose degradation, and reducing sugars utilization. In the present study, different ethanol yield was obtained due to different pretreatment procedure, different substrate and co-cultures technique were used. The present study showed the usefulness of SSF by co-culture strategy for maximum ethanol production from pretreated corn husk. The above results can be useful for commercial bioethanol production from cheaper agro-residues.

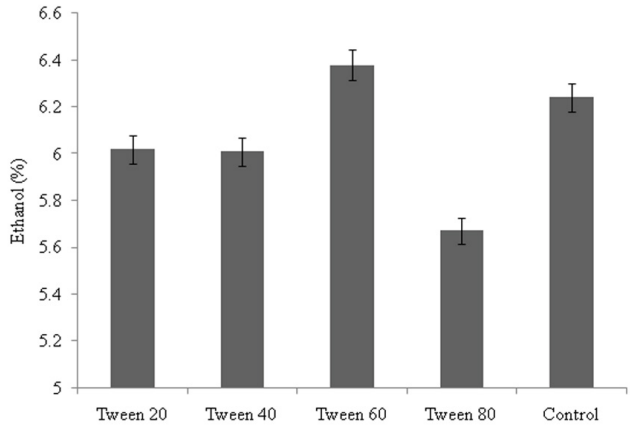

Figure 4: Effect of different non-ionic surfactants on SSF of pre-treated biomass.

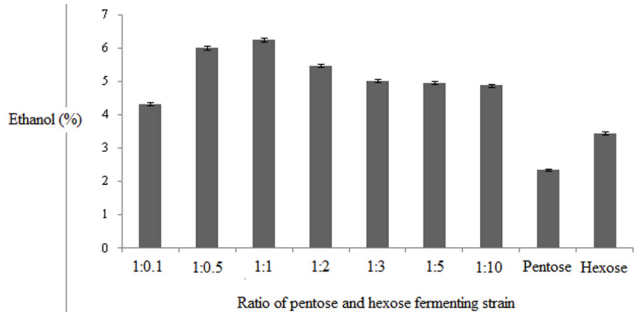

Figure 5: Responses for ethanol production under the influence of co-culture of variable concentration ratio of *Fusarium oxysporum* and *Saccharomyces cerevisiae*.

Conclusion

In the present study, simultaneous saccharification and fermentation (SSF) was carried out by co-culture strategy using thermochemically pretreated corn husk. Maximum ethanol production was observed after 24 h of incubation. Addition of surfactant (Tween 60) enhanced further ethanol production. Further, for additional enhanced ethanol production, different co-culture strategy was adopted. It was found that maximum ethanol production was obtained when sugar fermentation was carried out by *Fusarium oxysporum* followed by *Saccharomyces cerevisiae*. The present study can be useful for enhanced bioethanol production from lignocellulosic biomass.

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Conflict of Interest

The authors declare that they have no conflict of interest in the publication.

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