Indian Journal of Biotechnology
Vol. 18, July 2019, pp 253-259

Bioethanol production from pea hull aqueous extract as a novel substrate using *Saccharomyces cerevisiae*

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Received 31 January 2019; revised 27 March 2019; accepted 15 April 2019

Use of agricultural wastes as substrate for the production of bioethanol can help to solve environmental problems caused by inadequate discharge of waste. The production of bioethanol by *Saccharomyces cerevisiae* using pea hull aqueous extract as a substrate was evaluated in this work. Response surface methodology was employed to study the effect of different parameters such as inoculum concentration (3-7%), fermentation time (24-71 h) and pH (4.4, 4.8 and 5.2) on the production of bioethanol. Results revealed that highest bioethanol yield of 1.65% occurred at 6.8% inoculum concentration; 71 h of fermentation time in the medium with initial pH 5.2. The study highlights that pea hull aqueous extract can be utilized for bioethanol production with extended fermentation times.

Keywords: Bioethanol, fermentation, pea hull, *Saccharomyces cerevisiae*, agricultural waste.

Introduction

Demand for energy is increasing with rise in population and need of development. Biofuel is a major energy source which has great potential to reduce the load on fossil fuels. Blending of diesel with bioethanol has already been started in many countries like Italy, United States of America (USA), Canada, France, etc. Bioethanol or ethanol, a clear colourless sweet flavoured liquid, is a renewable source of energy and is a potential alternate to fossil fuels[1]. Commercially, ethanol produced by fermentation of sugar from conventional crops (sugarcane, corn etc.) is unable to meet the global demand of ethanol production due to its primary value of food and feed. Therefore, bioethanol production from lignocellulosic substances such as agricultural waste is an attractive avenue of research. Bioethanol production from agro-wastes, rich in lignocelluloses could be a promising technology; however, the conversion process of agro-waste to bio-ethanol can be challenging and is not devoid of limitations. Industrial lignocellulosic ethanol production is still a challenge due to its high processing cost. One reason for high cost is the high steam energy consumption in the distillation of fermentation broth with low ethanol titer when lignocellulose materials are used as feedstock[2]. Another reason for high cost in ethanol production is higher feedstock prices[3-5] whenever the substrate is non-cellulosic. So far different pretreatment methods have been developed to increase the cellulose content to upgrade ethanol titer[6]. The most promising technology for the conversion of lignocellulosic biomass of pea hull to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulase enzymes and pea hull could be a better carbon source for increasing the production of cellulase enzyme using fungal strain which produces cellulase itself. Nowadays, yeast is used to generate bioethanol from renewable energy resources. Nevertheless, economically speaking, ethanol production from lignocellulosic substrates using yeast strain such as *S. cerevisiae* keeps distillation cost low and gives a high ethanol yield, high productivity and can withstand high ethanol concentration[7]. Fermentation process carried out by yeast is known to vary with respect to various factors such as substrate concentration, temperature, pH, inoculum size etc. It is therefore, imperative to optimize the fermenting conditions for yeast cells so that production efficiency increases and finally the yield gets increased.

There are many reports on the production of bioethanol using agro-industrial wastes such as rice straws, wheatbran, fruits and vegetables peels as

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substrates[8-10] but no work has been reported on the bioethanol production from pea hull/hull juice. Therefore, the present study aimed to utilize pea hull/ hull juice as a substrate for developing optimized fermenting conditions for the production of bioethanol.

Materials and Methods

Substrate Preparation

Pea hulls (78.5% dry wt.) were procured from KLA Frozen Fruits & Vegetables Industry, Rudrapur, Uttarakhand and stored at 4°C until further use. Pea hull slurry was prepared by grinding the hulls (hulls to water ratio of 1:1) at 18,000 rpm for 3 min in a blender (Sujata, India). After grinding, the slurry was heated at 65°C for 5 min to get a clear suspension[11], which was filtered through a filter paper (Filter-Lab 1249) to remove the solids. The clear suspension was collected and sterilized at 121°C for 20 min.

Microorganism and Inoculum Preparation

Yeast (*Saccharomyces cerevisae*) strain was obtained from the Department of Biological Sciences, College of Basic Sciences and Humanities, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. It was grown on potato dextrose broth (PDB) by incubating at 28 ± 2°C for 24 h and preserved on potato dextrose agar (PDA) plates by growing at 28 ± 2°C for 24 h (Fig. 1).

Fermentation Experiments

Initial pH (4.4, 4.8, 5.2) of the medium, inoculums concentration (3%, 5% and 7%), and fermentation time (24, 48 and 72 h) were chosen for fermentation studies as most influencing parameters based on experimental pre-trials. Fermentation experiments were conducted employing Box-Behnken design of experiments. Briefly, 200 mL of sterilized pea hull aqueous extract (with set experimental pH using 1N HCl) was placed in a 500 mL conical flask, inoculated by yeast and incubated at 28 ± 2°C for 71 h. Samples were withdrawn as whole flask (in duplicate) at 24, 48 and 71 h for determination of total sugars, reducing sugars and bioethanol yield.

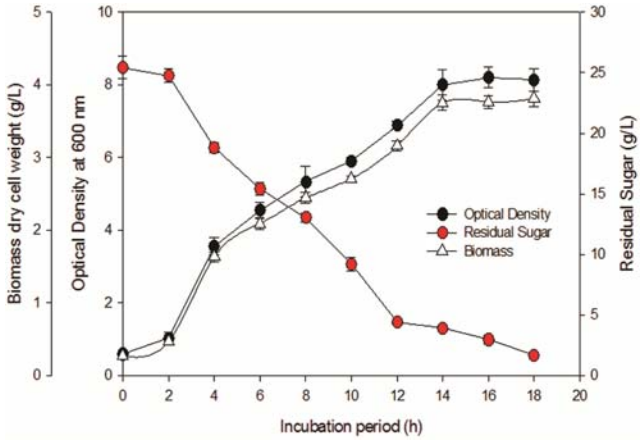

Fig. 1 — Typical growth curve of *S. cerevisiae.*

Estimation of Total and Reducing Sugars

Total sugars and reducing sugars were estimated by phenol-sulfuric acid method and DNS (3,5-dinitrosalicylic acid) method, respectively[12-13]. Briefly, 5.0 mL of sample was centrifuged at 4°C and 6000 x g for 10 min. Pellet was discarded and supernatant was used for estimation of sugars. For total sugars, 1.0 mL of 5% (w/w) phenol and 5.0 mL of 96% sulfuric acid were added to 1.0 mL of supernatant and mixed well. Absorbance was measured at 490 nm after 30 min of incubation at room temperature (25°C). For reducing sugars, 1.0 mL sample was taken to which 3.0 mL of freshly prepared DNS reagent was added. The contents were thoroughly mixed in a test tube and placed in a boiling water bath at 90°C for 10 min. The tubes were rapidly cooled to room temperature. The concentration of reducing sugars in the sample was estimated by computing the absorbance at 510 nm against the standard curve of glucose. Results were expressed in mg/mL glucose. Glucose standard of different concentration (0 to 20 mg/mL) were prepared for generating the standard curve. Results were expressed in mg/mL glucose.

Estimation of Bioethanol by Gas Chromatography (GC)

Ethanol standards of different concentrations (1-10%, w/v) were prepared. The standard solutions and fermented samples were filtered through a 0.22 µ filter paper and 1 µL of each sample was injected separately into the injector of the GC apparatus (7890A, Agilent Technologies, USA) using a syringe. Helium was used as the carrier gas. The injector temperature was set at 200°C with an initial column temperature of 65°C and final temperature of 150°C. The detector temperature was kept at 250°C. Total programme time was 20 min with sample holding time of 5 min. The retention time, peak height and peak area were recorded. Chromatograms of the sample solution and the standard were compared for ethanol estimation[14] (Fig. 2).

Statistical Analysis

Analysis of variance (ANOVA) was performed over the experimental data at 5% level of significance

using Design Expert software v.10.0.1 (Stat-Ease Inc., USA). Second order regression models were determined for the responses as per Eqn. [1]:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 \quad \ldots (1)$$

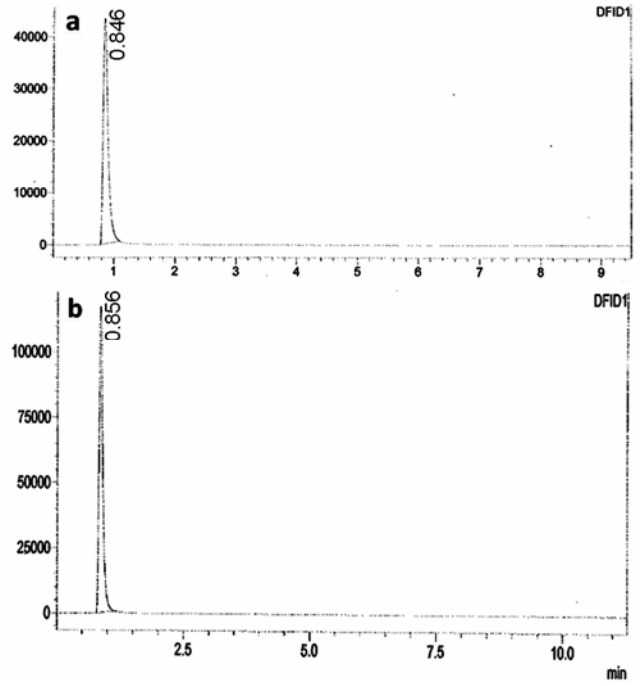

Fig. 2 — Gas chromatograms of 5% (a) and 10% (b) pure ethanol.

Where, β_0, β_i, β_{ii}, β_{ij} are the coefficients of intercept, linear, quadratic and interaction variables respectively; $X_{i,j}$ represents the independent variable and *Y* represents the response.

Results and Discussion

Table 1 shows the responses obtained for this study. Non-linear second order models were developed between the independent variables: inoculum concentration (X_1), fermentation time (X_2) and pH (X_3), and response (Y). Model parameters obtained for the responses are shown in Table 2.

Total Sugars Consumption

Pea hull aqueous extract contained 16.5 mg/mL total sugars. During fermentation, yeast culture consumed 80-90% total sugars (13.06 to 14.53 mg/mL). The maximum sugar consumption (14.53 mg/mL) was observed at an inoculum concentration of 5%, 72 h fermentation time and a pH of 5.2. On the contrary, lower values of inoculum concentration (3%), fermentation time (24 h) and pH (4.8) showed minimum total sugar consumption (13.06 mg/mL). Fermentation time and pH showed pronounced effect on total sugars consumption ($p < 0.05$) (Table 2). Since, a longer fermentation time could allow for formation of suitable size of yeast colony, sugar consumption would be proportional to the size of the colony[15]. However, optimum pH must be maintained in

Table 1 —.Experimental results at different conditions for fermentation of pea hull juice

Run no.	X_1:Inoculum conc. (%)	X_2:Fermentation time (h)	X_3:pH	Total sugar consumed (mg/mL)	Reducing sugar consumed (mg/mL)	Bioethanol yield (%)
1	5	24	5.2	13.24	3.53	1.44
2	7	24	4.8	13.33	2.95	1.23
3	5	24	4.4	13.34	2.24*	1.02
4	7	48	5.2	13.94	3.59	1.42
5	5	72	4.4	1.53	2.84	1.28
6	5	48	4.8	13.65	3.13	1.31
7	5	48	4.8	13.57	3.24	1.4
8	7	48	4.4	13.61	2.48	1.04
9	7	72	4.8	14.01	3.75**	1.62**
10	5	48	4.8	13.54	3.29	1.44
11	5	48	4.8	13.74	3.11	1.33
12	5	72	5.2	14.53**	3.69	1.54
13	3	48	5.2	13.42	3.32	1.17
14	3	48	4.4	13.44	2.41	0.97*
15	3	72	4.8	14.03	3.06	1.11
16	3	24	4.8	13.06*	2.63	1.01
17	5	48	4.8	13.48	3.09	1.3

*minimum, **maximum

Table 2 — Regression coefficients of second order models obtained for each response of pea hull juice fermentation

Source	Total sugar consumed (mg/mL)		Reducing sugar consumed (mg/mL)		Bioethanol yield (%)	
	Coeff.	*p*-value	Coeff.	*p*-value	Coeff.	*p*-value
Model	13.60	0.0013**	3.17	0.0003**	1.36	0.0021**
X_1: Inoculums conc.	0.12	0.1342	0.17	0.0076**	0.13	0.0016**
X_2: Fermentation time	0.39	0.0001**	0.25	0.0009**	0.11	0.0051*
X_3: pH	0.15	0.0118*	0.52	0.0001**	0.16	0.0006**
X_1X_2	-0.073	0.2898	0.092	0.1945	0.072	0.0942
X_1X_3	0.087	0.2095	0.050	0.4635	0.045	0.2687
X_2X_3	0.27	0.0034**	-0.11	0.1318	-0.040	0.3211
X_1^2	-0.023	0.7204	-0.100	0.1565	-0.14	0.0060**
X_2^2	0.034	0.5936	0.025	0.6998	0.028	0.4644
X_3^2	0.030	0.6472	-0.12	0.0928	-0.064	0.1219
R^2	94.37%		96.37%		93.55%	
Adj. R^2	87.14%		91.70%		85.25%	

**,*, significant at 1, 5 level of significance

order to proliferate yeast cells. Hence, pH, in addition to fermentation time, had a bearing on the total sugars consumed. Model parameters also indicated a significant ($p < 0.05$) synergistic effect between fermentation time and pH (Table 2). These results indicated high model precision ($R^2 = 0.94$) in predicting the total sugar consumption within the experimental range of variables. Table 2 showed that the developed model for total sugar was able to explain 94.37% of the total variation in experimental results.

Total sugars consumption was more sensitive towards fermentation time as compared to pH. This was observed from a higher significance in the model coefficient corresponding to fermentation time than pH. Since, yeast is an acidophilic organism[16], the effect of pH would have a relatively lower impact as compared to other variables given the experimental boundaries (pH = 4.4 to 5.2) of this investigation. Amadi and Ifeanacho[17] reported significant effect of fermentation time on the sugar consumption by *S. cerevisiae* during fermentation of lignocellulosic biomass. Hashem *et al*[18] also reported that fermentation duration upto 71 h resulted in higher production of ethanol due to efficient utilization of sugars.

Increase in inoculum concentration and pH had a positive influence on the total sugar consumed ($p < 0.05$), probably due to increased yeast cell concentration leading to better sugar consumption. The interaction effect of inoculum concentration with pH and fermentation time, however, was non-significant. Moreover, none of the variables contributed significantly to any kind of non-linearity. This explained the fact that the total sugar consumed increased at higher levels of independent variables.

Reducing Sugar

Initial reducing sugar content in the substrate was estimated at 4.27 mg/mL. During fermentation, the consumption of reducing sugar ranged from 2.24 to 3.75 mg/mL. Maximum consumption of reducing sugar (3.75 mg/mL) was observed for experimental conditions of 7% inoculum concentration, 71 h fermentation time and initial pH of extract as 4.8, while minimum reducing sugars consumption (2.24 mg/mL) was observed for the treatment of 5% inoculum concentration, 24 h fermentation time and medium pH of 4.4. The effect of fermentation time and inoculum concentration on reducing sugars was significant ($p < 0.05$). Higher inoculum concentration and fermentation time resulted in more consumption of reducing sugars. Similar results were reported by Akinosanaiye *et al*[19]. On papaya fruit waste fermentation using yeast strain. Higher inoculum concentration allows for greater reducing sugar consumption by means of higher metabolic activity that is established by virtue of greater cell number[20].

Bioethanol Production

During fermentation, the ethanol yield ranged from 0.97 to 1.62% (w/v). The maximum ethanol yield (1.62%) was observed at an inoculum concentration of 7%, fermentation time of 71 h and pH of 4.8, while the minimum ethanol yield was observed for the experiment with inoculum concentration of 3%, fermentation time of 48 h and initial medium pH of 4.4. It was observed that higher levels of experimental variables resulted in higher ethanol yield. The results were consistent with the findings of Laopaiboon *et al*. who stated that inoculum concentration significantly affected the ethanol productivity[21]. Ethanol yield may

be considered proportional to the inoculum concentration when both fermentation time and pH are uniformly increasing (i.e., increasing by equal amounts after equal intervals of time). This complies with the logic that a greater cell size can yield enough ferment, only when maintained for a time sufficient for the enzymatic reaction to happen and, at an optimum pH for those metabolic reactions to occur[22]. As the fermentation time increases, the bioethanol yield mandates an adjustment in pH for equilibrium to be broken and reactions to proceed again in the forward direction. The pH of the media also influences the bioethanol production due to cell growth of the microorganism depending on the initial pH of the medium. Higher pH (5.2 or above) results in production of acetic acid and glycerol which can inhibit the fermentation of ethanol[23-24]. However, it is evident from the Table 1 that both time and pH increased uniformly for respective increase in yield to occur. Moreover, experimental data suggested that a greater change in yield occurred when the pH was fixed and the fermentation time was increased. This meant that among the two (fermentation time and pH), bioethanol yield was more sensitive to fermentation time.

The statistical significance of linear, interactive and quadratic terms for bioethanol yield is presented in Table 2. Model obtained for prediction of bioethanol yield explained 93.55% of the linear variation and 85.25% of non-linearity in data. Based on model parameters, it was inferred that the inoculums concentration and pH affected the yield significantly ($p < 0.01$), followed by fermentation time ($p < 0.05$). Change in inoculum concentration resulted in non-linearity in data which indicated that bioethanol yield would increase only up to a particular level of inoculum concentration. This was also evident from Figure 3a, which showed that the ethanol yield attained a constant value at an inoculum concentration ~6.5%; beyond this level, the yield remained constant or even decreased. This showed that inoculum concentration was the deciding variable for obtaining an optimum yield of bioethanol. Increase in the fermentation time, however, increased bioethanol yield indefinitely (Fig. 3b). Capping the maximum value of fermentation time was important to avoid any unnecessary increase in the processing time. The effect of pH on bioethanol yield was found similar to that of inoculum concentration (Fig. 3c). It is noteworthy to mention that the highest chosen level of pH nearly approaches the maximum possible yield of bioethanol. A higher pH level upto 5.5 could increase the bioethanol yield marginally.

Optimization of Parameters

For optimization, goal for factors as well as responses namely total sugars, reducing sugars and bioethanol yield were selected. In the optimization algorithm, the constraints for the independent variables (inoculum concentration, fermentation time and pH) were within the experimental range whereas the responses were set to maximize. Based on the mentioned criteria, a numerical optimization was carried out. After optimization, 100 solutions were generated out of which the optimum condition with the highest desirability was selected. The optimum level of independent variables in coded and actual form is shown in Table 3. Evidently, the optimum fermenting conditions providing the maximum bioethanol yield of 1.65% were inoculum concentration as 6.8%, fermentation time as 71 h and initial pH of the medium (aqueous extract) as 5.2.

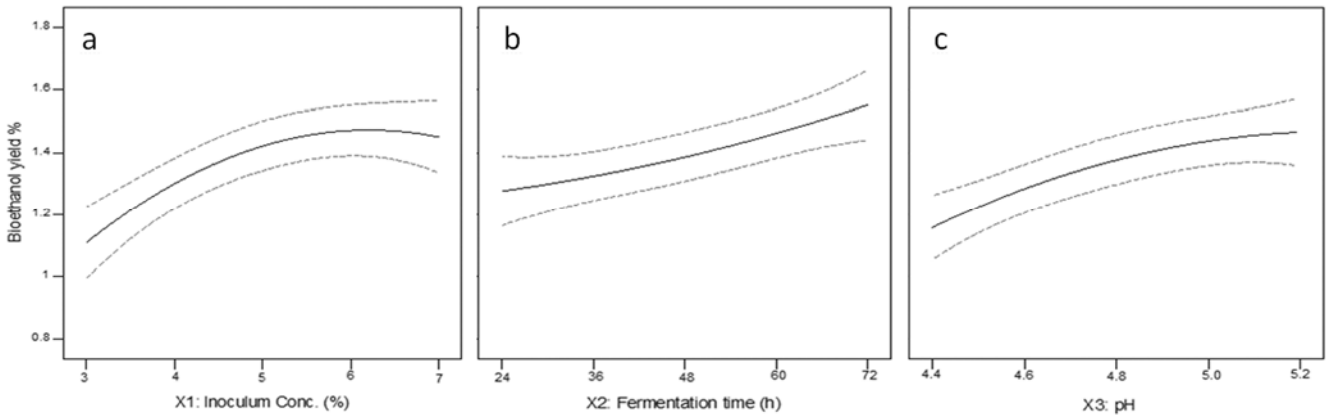

Fig. 3 — Variation in ethanol yield with increase in (a) inoculum concentration, (b) fermentation time, and (c) pH.

Table 3 — Optimized pea hull juice fermentation process conditions and responses

Independent variables	Optimum values		Responses	Optimum values
	Coded	Uncoded		
Inoculum conc.(%)	0.913	6.8	Total sugar consumed	14.55 mg/mL
Fermentation time (h)	0.961	71	Reducing sugar consumed	3.92 mg/mL
pH	0.991	5.2	Ethanol yield	1.65%

Validation of Optimized Parameters

To validate the accuracy of the generated model and optimum results, a confirmatory experiment with duplicate sets was performed. Verification was done by comparing the predicted values for the responses with that of the experimental results. The predicted total sugars, reducing sugars and bioethanol yield suggested by the software under the optimized conditions were 14.55 mg/mL, 3.92 mg/mL and 1.65%, respectively. The actual values were in close agreement with the predicted values with a maximum difference of 4%. According to Levin *et al*[25]. differences between the experimental and predicted values of less than 10% confirms the validity of a model. Hence, the developed model from response surface methodology in the present study can be considered consistent and reproducible.

Conclusion

From the results it could be concluded that pea hull aqueous extract could be used for the production of bioethanol using *S. cerevisiae*. Use of lignocellulosic waste for the production of bioethanol production offers potential for environmental sustainability. However, better yield also depends somewhat on selection of microorganisms, fermentation mode and techniques that influence fermentation factors. The present investigation could establish that pea hulls which have not been exploited commercially for any industrial application and are poorly disposed off, could effectively be used for ethanol production through fermentation using yeast strain. Supplementation of additional nutrients and increase in fermentation time could be effective in increasing the bioethanol yields.

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