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Materials Today: ProceedingsXX (2016) XXX–XXX

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PMME 2016

Experimental Investigation and Optimization for Production of Bioethanol from Damaged Corn Grains*

Sheetal B. Gawande[a], Indrasing D. Patil[b]*

[a]*Research Student, SSBT`s, COET, Bambhori, Jalgaon - 425001, MS, India & Assistant Professor, Department of Food Technology, Laxminarayan Institute of Technology, Nagpur*
[b]*Professor and Head, Biotechnology Department, SSBT`s, COET, Bambhori, Jalgaon 425001, MS, India*

Abstract

Bioethanol production from damaged corn grains provides an alternative for bio-refineries. Studies on Simultaneous Saccharification and Fermentation (SSF) of damaged corn grains flour as substrate using symbiotic strains of starch digesting Aspergillus niger (NCIM 1248), non-starch digesting and sugar fermenting Saccharomyces cerevisiae (MTCC 170) in a batch fermentation are carried out. Statistical experimental design is used for optimization of process variables for ethanol production. Experiments based on Central Composite Design (CCD) are conducted to study effects of substrate concentration, pH, temperature and their different combinations on Ethanol Concentration. These parameters are optimized using Response Surface Methodology (RSM). To determine optimum response, surface plots for desirability and overlay plots are generated. Optimum conditions obtained are pH (5.6), temperature (31^0C) and substrate concentration of (14 %) to get maximum ethanol concentration of 4.24 (g/100ml) after 48 hours with ethanol productivity 0.88 (g/l/h). The SSF of damaged corn grains flour under optimized conditions represented about 69 % of total ethanol production, when fine corn grains flour 6.3 (g/100ml) was used as substrate. This work demonstrates that ethanol yield can be enhanced by optimization of process variables from damaged corn grains.

Selection and Peer-review under responsibility of International Conference on Processing of Materials, Minerals and Energy (July 29th – 30th) 2016, Ongole, Andhra Pradesh, India.

*Keywords:*Optimization; Damaged Corn Grains; Response Surface Methodology; Bioethanol

* Corresponding author. Tel.: + 919404492192

*E-mail address:*sheetalddeshmukh11@gmail.com

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1. Introduction

Bioethanol has stimulated worldwide interest due to its utilization as an alternative fuel source and is produced from renewable cheap agricultural resources. Bio-ethanol is a fuel derived from biomass sources of feedstock; typically plants such as wheat, sugar beet, corn, straw, and wood. Bio-ethanol can be produced from different kinds of raw materials. These raw materials are classified into three categories of agricultural raw materials: simple sugars, starch and lignocellulose. Conversion technologies for producing bio-ethanol from cellulosic biomass resources such as forest materials, agricultural residues and urban wastes are under development and have not yet been demonstrated commercially [1]. Renewable bioresources are available globally in the form of residual agricultural biomass and wastes, which can be transformed into liquid biofuels. However, the process of conversion, or chemical transformation, could be very expensive and not worth-while to use for an economical large-scale commercial supply of biofuels. There is still need for much research to be done for an effective, economical and efficient conversion process [2].

Corn has the potential of becoming a useful energy crop. However, fresh starchy materials are required for human consumption. Starchy grains like corn is the main source of caloric intake in Latin America and some areas of Africa. Therefore its use for biofuel is not socially practicable. About 5% of corn in the world is wasted. If wasted corn could be fully utilized as feedstock for producing bioethanol, then 9:3 GL of bioethanol could be produced, thereby replacing 6:7 GL of gasoline if bioethanol is used as an alternative vehicle fuel, E85 [3].

A large quantity of different grains is spoiled every year in India because of unfavourable climatic conditions and inadequate transport and storage facilities. Main causes of grain damages are mechanical damages during harvesting and handling. Moisture content and due to insecticides deterioration of grain occurs heavily. After identification starchy grains in damaged condition, the next best option for its utilization is as fuel energy production [4]. Use of insect, fungi and sprout-damaged grain in other industrial processes could reduce, at least to some extent, the producer losses.

Regarding use of damaged kernels in ethanol production, Yan et al. [5] tested field-sprouted-sorghum and concluded that use of these kernels significantly reduced fermentation time and yielded higher ethanol. Chuck-Hernandez et al. [6] investigated the bioconversion into ethanol of insect (Sitophilus zeamais), mold (Aspergillus flavus) and sprout damaged maize and sorghum. Their research demonstrates that the use of already damaged insect, mold or sprouted kernels is feasible and a good alternative for biorefineries. Although there are reports on ethanol production from damaged grains, little information is available on its optimisation.

The production of industrial and fuel ethanol commonly involves three steps: 1) liquefaction of starch by α-amylase, 2) enzymatic saccharification of liquefied products to produce glucose and 3) fermentation of glucose to ethanol. Commercial enzyme glucoamylase is used for sachharification and represent a significant expense in the production process. This study aims at eliminating the enzymatic saccharification step by using coculture of amylolytic and sugar fermenting organisms to maximize the ethanol yield from starch. Simultaneous saccharification and fermentation (SSF) of starch with an amylolytic mold and yeast is an efficient and economical method for ethanol production due to lesser equipment cost.

Response surface methodology (RSM) is an important statistical technique employed for multiple regression analysis using quantitative experimental data obtained from properly designed experiments using central composite design (CCD). The response surface is a two dimensional graphic representation to study individual, interactive, and cumulative effects of variables. Mathematical models play an important role in rational design and optimization of biochemical process. It is difficult to obtain an accurate model for biochemical process such as ethanol production by coculture method due to the inherent complexity.

In present study a central composite design (CCD) of response surface methodology (RSM) has been used for optimization of the process parameters in simultaneous saccharification, and fermentation of damaged corn grains flour to ethanol using coculture of Aspergillus niger (NCIM 1248) and Saccharomyces cerevisiae (MTCC 170).

2. A Review on Utilization of Damaged Corn Grains

Main causes of grain damages are mechanical damages during harvesting and handling grain size reduces due to breakage. Moisture content and due to insecticides deterioration of grain occurs heavily. After identification of

sorghum and corn grain in damaged condition, that is these grains are not usable as food or feed. The next best option for its utilization is as fuel energy production [4]. Sprouted, insect and mold damaged Corn and Sorghum grains have some influence on the process or end products. Though broken grains are not of major concern for ethanol production, sprouted, insect, and mold damaged Corn and Sorghum grains can have a considerable effect on ethanol yield. Nevertheless, all already damaged kernels had similar fermentation efficiencies, indicating that these feedstocks are suitable for fuel ethanol production. Field sprouted grains had more rapid fermentation rate than healthy grains. Field sprouted grains shorten fermentation time without decreasing ethanol yield. Low test weight is considered to reduce ethanol yield because the kernels are not as densely packed with starch. As per the above study, it is concluded that already damaged insect, mold or sprouted corn and sorghum grains could be effectively used for ethanol production and can be a good alternative for bio-refineries, instead of using them for animal feed or manure which is unhealthy. Ethanol production using damaged grains is an economical method of using waste to produce valuable products [7].

Generally, economic restrictions force industrial processes to work in a very small range of operating conditions. Mathematical models are effective tool for analyzing biological process and microbial growth phenomenon. The studied model shows more insight into the environmental conditions that is surrounding bio-process and can be used for further development and optimization of bio-processes. This paper reviews the various process options and kinetic models adopted towards resolving the technological challenges to develop a low-cost commercial process. Optimization of the cost of ethanol production is the prime objective of the current research work. In this direction improvement of yield and productivity are the major achievements to reduce the cost of ethanol production per gallon. Kinetics of biomass production with respect to time could be illustrated by logistic models. Kinetics of ethanol mass concentration production at an operating temperature could be tested by modified kinetic models. Logistics model could be well fitted to the experimental data and could be regarded as sufficient to describe the biomass production. Also, the modified kinetic model fitted into the experimental data and could also be regarded as sufficient to illustrate the fermentation process for the production of ethanol from glucose biomass with a novel thermo-tolerant strains techniques. Experimental and mathematical modelling, results were compared by several researchers which shows no significant difference. Therefore, utilization of mathematical model would contribute to a better understanding of effects of various factors affecting the production of ethanol. In other words, models enable us to understand, design and control the fermentation process better and could be also be used for further process development [8]. A significant progress and enhancement in the economy of bioethanol production on starch--based raw materials may be obtained by the process optimization of feedstock pre-treatment and ethanol fermentation itself, and by an adequate utilization of the process by-products. The introduction of new pre-treatments such as microwave and ultrasound can improve the starch gelatinization process, the substrate susceptibility to enzymes and greatly influence and improve the effects of hydrolysis and subsequent ethanol fermentation. In the domain of fermentation, the choice of the production microorganism, media optimization, and the choice of the most appropriate process flow sheet (simultaneous saccharification and fermentation, utilization of immobilized yeasts, etc.) are significant for the development of an efficient production process. Thus, utilization of especially damaged cereal grains for bioethanol production is the best option for fossil fuel shortage [9].

Damaged corn and sorghum grains has immense potential source for non-edible option due toremoval waste part as well as constituents reducing bio-ethanol conversion from it. Use of techniques available for separation of damaged portion would further enhanced ethanol production yield from corn and sorghum starch. Complete process optimization from damaged corn and sorghum grains is possible by the study of each step involve during conversion from damaged grains to ethanol. So, more stress is given on study of bifurcation useful and nonproductive material in this work which is not specifically focused earlier [10]. Minimization of the cost of ethanol production is the prime objective of the current research work. In this direction improvement of yield and productivity are the major achievements to reduce the cost of ethanol production per gallon. Kinetics of biomass production with respect to time could be illustrated by logistic models. Kinetics of ethanol mass concentration production at an operating temperature could be tested by modified kinetic models. Logistics model could be well fitted to the experimental data and could be regarded as sufficient to describe the biomass production. Also, the modified kinetic model fitted into the experimental data and could also be regarded as sufficient to illustrate the fermentation process for the production of ethanol from glucose biomass with a novel thermo-tolerant strains techniques. Experimental and

mathematical modelling, results were compared by several researchers which shows no significant difference. Therefore, utilization of mathematical model would contribute to a better understanding of effects of various factors affecting the production of ethanol. In other words, models enable us to understand, design and control the fermentation process better and could be also be used for further process development [11].

3. Materials and Methods

3.1. Substrate

Study is carried at Laxminarayan Institute of Technology, Nagpur, MS, India. Damaged corn seed grains were obtained from local market. The grains had 40% sound and 60% damaged grains, categorized as industrial use [12, 13]. Damage corn grains includes broken, cracked, and attacked by insects, dirty, discoloured. Composition of damaged grains used is given in table 1. Grains were washed with water, sun dried and then powdered. Fine corn grains flour is obtained from flour mill and used to control experiments further comparing with damaged grains.

Table 1. Composition (% W/W) of fine and damaged (60%) corn grains.

Composition	Fine Corn grains	Damaged Corn grains
Starch	62	45
Reducing sugar	6.2	5.8
Crude Protein	9	7.8
Crude Fibre	2.9	2.2

3.2. Microorganisms

Amylase-producing fungus A. niger (NCIM 1248) was obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, MS, India. Stock cultures were maintained on potato dextrose agar slants. A standard strain of S. cerevisiae MTCC 170 was obtained from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India for using as a control organism. Strains were maintained on slants of YPD agar medium.

3.3. Media

Yeast strain *S. cerevisiae* is maintained on YPD medium containing 0.5% yeast extract; 0.5% peptone; 2% dextrose; 2% agar; pH 5.5. Yeast culture is grown for inoculums development in a controlled environment shaker (150 $r.min^{-1}$) at 35°C for two days in liquid medium containing glucose 2%; peptone 0.5%; yeast extract 0.3%; KH_2PO_4 0.1%; $MgSO_4$ $7H_2O$ 0.05%; pH 5.5. Growth medium used for preparing the fungal inoculum contained soluble starch l%, peptone 0.2%, yeast extract 0.2%, $MgSO_4$ $7H_2O$ 0.l%, $(NH_4)_2PO_4$ 0.2%. The fermentation medium used for ethanol production from starch was identical to growth medium except that the substrate used is damaged corn grains powder. 1 N HCl or 1 N NaOH was used to obtain desired pH for testing the effect of pH on fermentation.

3.4. Pretreatment of Damaged Corn Grains Flour

Damaged corn grains flour was gelatinized in an autoclave at a pressure of 15 Psi for one hour. The solution was cooled and pretreated using fungal amylase enzyme obtained from Hi media laboratories for an hour. Temperature is maintained at 60 °C in a constant temperature water bath and pH at 6 using phosphate buffer.

3.5. Simultaneous Saccharification and fermentation

Saccharification and fermentation of raw starch is carried out simultaneously in a Flask. Crude amylase broth (10 ml) of A. niger is dispensed into 500 ml Erlenmeyer flasks with sterile fermentation medium containing various concentration damaged corn grains flour. The medium was inoculated with *S. cerevisiae* suspension (1x 10^9 cells/ml)

and incubated for 5 days with shaking at 150 rpm. Operating conditions of medium substrate concentration, pH and temperature maintained during fermentation are kept at three different levels as shown in table 2. Since starch is insoluble in water at room temperature initial broth gave viscous slurry. Viscosity of medium decreased rapidly due to the saccharification and fermentation. All experiments are replicated twice and average values are obtained. Variation of about 5% is observed between two experiments.

4. Analytical Procedures

Amylase activity is assayed by reducing sugars released from starch. The reaction mixture containing 2 ml of 1% starch in deionized water, 1 ml of 0.1 M acetate buffer (pH 5.8) and 1 ml of enzyme solution is incubated at 35°C in water bath for 10 minutes. After incubation the amount of reducing sugars are estimated. Estimation of total reducing in enzymatic hydrolysate of damaged sorghum grains flour is done by DNS method [14]. The estimation of ethanol was done by spectrophotometer [15]. The ethanol volumetric productivity (g/l/h) was calculated as ratio of ethanol concentration (g/l) at the end of the run to fermentation time (t, h).

5. Experimental Design and Optimization

Factors affecting the ethanol yield from damaged grains flour using a coculture of A. niger and S. cerevisiaewere studied using CCD experiments. Values of pH, temperature (°C) and substrate concentration (%) were chosen as independent variables and is shown in table 2. Ethanol production (g/100ml) is used as the dependent output variable. Twenty experiments based on the CCD were carried out with different combinations of variables, and results were presented in table 3. A second order polynomial model is predicted with DOE (equation 1) indicating linear, interaction and quadratic effect of variables on system response as either + ve or - ve. ANOVA analysis of the model is performed to evaluate its statistical significance as shown in table 4.

Table 2. Factors with their coded levels

Sr. No.	Variable	-1.682	-1	0	1	1.682
1	pH	4.5	5	5.5	6	6.5
2	Temperature	26	28	30	32	34
3	Substrate Conc (%)	4	8	12	16	20

Table 3. Three-level central composite design and experimental response

Sr. No.	Factor Variables			Response Variable
	pH	Temperature	Substrate Conc. (%)	Ethanol conc. (g/l)
1	0	0	0	4.12
2	-1.682	0	0	0.50
3	0	0	1.682	3.81
4	0	1.682	0	3.85
5	0	0	-1.682	3.32
6	1	1	-1	3.32
7	1	-1	-1	3.00
8	1	-1	1	3.21
9	-1	1	1	2.60
10	0	0	0	4.12
11	-1	-1	-1	2.14
12	0	-1.682	0	2.70
13	0	0	0	4.12
14	-1	-1	1	2.16
15	0	0	0	4.12
16	-1	-1	-1	2.40
17	0	0	0	4.12

18	1	1	1	3.59
19	0	0	0	4.12
20	1.682	0	0	1.41

6. Results and Discussions

1. Optimization of process parameters for bioethanol production is carried out. CCD matrix with ethanol production via response surface method predicts response as a function of three variables and their interactions in terms of their coded values. The CCD matrix with response is presented in table 3. A second order polynomial model fit to the experimental data for optimizing as follows.

$$Ethanol\ conc = 4.1 + 0.39\,pH + 0.24\,Temperature + 0.11\,Substrate\ conc. - 1.056\,pH * pH - 0.23\,Temperature * Temperature - 0.13\,Substrate\ conc * Substrate\ conc + 0.0001\,pH * Temperature + 0.03\,pH * Substrate\ conc. + 0.03\,Temperature * Substrate\ conc. \quad (1)$$

ANOVA calculations listed in table 4 shows F and P values obtained from model. Analysis of variance (ANOVA) is carried out for ethanol production. This gives good correlation between input factors and their responses. Analysis of variance (refer table 4) and significance of term coefficients (refer table 5) for ethanol content are determined.

ANOVA of regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test with a very low probability value [(p model > F) = 0.001]. The p-value denoting significance of coefficients was also important in understanding the pattern of mutual interactions between variables. Goodness of the fit of model is checked by 'determination coefficient' R^2, the value of R^2 and adjusted R^2 are 0.968 and 0.932 respectively, which shows a high correlation between observed values and predicted values. This means that regression model provides an excellent explanation of the relationship between independent variables (factors) and the response (ethanol production). No abnormality was observed from the diagnoses of residuals. Thus, it can be concluded that the model was statistically sound.

2. Response surface methodology for two variable Interaction studies is done. Surface plots for different interaction of any two independent variables, while holding third variable constant, on ethanol production are generated using Minitab software. Graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable in order to deduce optimum conditions. A direct correlation is found between substrate concentration and temperature on ethanol production at fixed pH 5.5 as shown in figure 1. As observed in the plot, increase in substrate concentration increased the concentration of ethanol. However, increasing concentration beyond certain level in the solution affected the ethanol production. As per figure 1, it could be observed that at any designated level of substrate concentration, ethanol concentration increased proportionally with increase in temperature to about 30^0C. Increase in temperature above 30^0C did not significantly enhance the ethanol production.

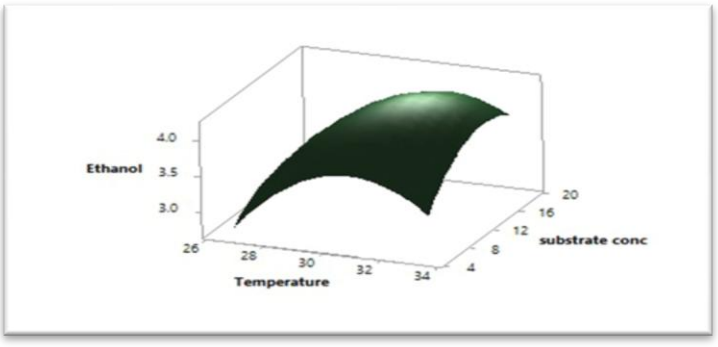

Fig. 1. Surface plots showing effect of substrate concentration and temperature on ethanol concentration

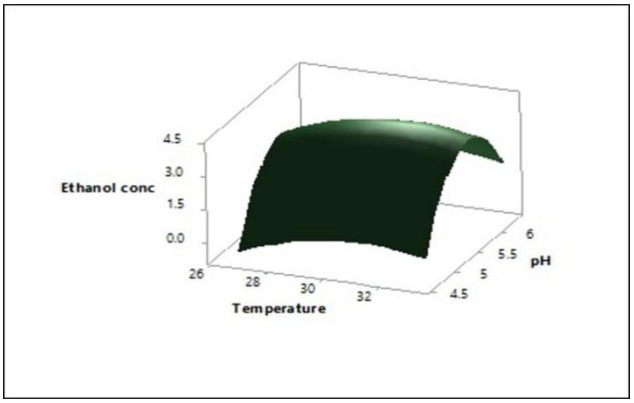

Fig. 2. Surface plots showing effect of temperature and pH on ethanol concentration.

Figure 2 shows surface plots of pH and temperature at fixed substrate concentration of 12%. Since ethanol is volatile, temperature has intense effect on ethanol production. A lesser amount of ethanol production is found at 20^0C temperature but it gradually increased up to 30^0C. The yield of ethanol is found to be maximum at this temperature. Above 30^0C temperature ethanol yield is observed to be decreased. Temperature between 28^0C and 30^0C has been usually employed for culturing yeast and temperature above 30^0C has been found to cease ethanol fermentation due to yeast growth inhibition at higher temperature [16].

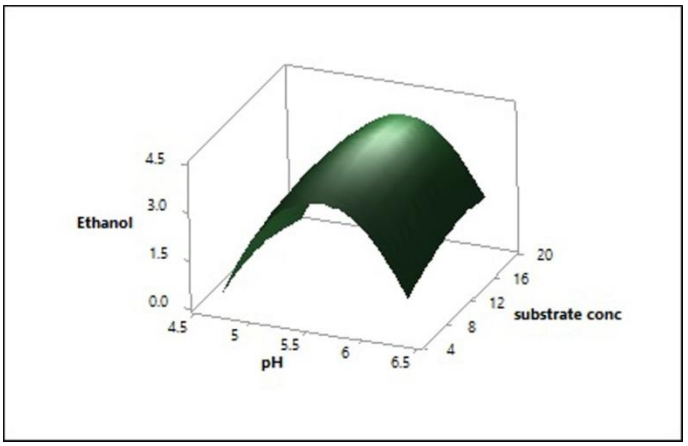

Fig. 3. Surface plots showing effect of substrate concentration and pH on ethanol concentration

Figure 3 shows surface plots of substrate concentration (%) versus pH at fixed temperature 30^0C. The concentration of ethanol augmented with increase in pH up to 5.8. Further increase in pH was not effective in promoting ethanol concentration. The pH is very important for enzyme activity, pH influences the metabolic activity of organism and as the pH level increases ethanol production also increases. The pH also has a strong positive effect on the biotechnological process because value of pH affects both enzyme activity and yeast fermentation [17].

The starch content in damaged grains used was lower by 30% and 40% when compared with fresh grains of corn (refer table 1). But as these damaged grains are cheaper by 10 times than fresh grains, it would still be cheaper to utilize them for ethanol production using co-culture. In this paper efforts are made to optimize ethanol production process from damaged corn grains with specific objective to study process parameters of ethanol production through fermentation by utilizing damaged corn grains flour. Suresh *et al*. [12] developed a simultaneous saccharification and fermentation system for producing ethanol from damaged sorghum (50% sound and 50% damage grains). They have reported ethanol yields of 91.5% and 78.6% of the theoretical ethanol yield with use of VSJ1 strain and

standard strain MTCC 170 for damaged sorghum grains. These authors later utilized a similar SSF method to compare ethanol production from damaged (50% sound and 50% damaged grains) and high quality sorghum [13]. A. Singh and N.R. Bishnoi [18] studied enzymatic hydrolysis by crude unprocessed on-site produced enzymes. They successfully employed response surface methodology to optimize ethanol production medium and process variables for ethanol production by S. cerevisiae using microwave alkali pretreated rice straw. They have reported maximum ethanol concentration of 13.2 g/L with ethanol productivity 0.33 g/L/h under optimum conditions of inoculum level 3%, pH 5.75, temperature 30^0 C and urea concentration 0.50 g/L according to the developed model.

Table 4. ANOVA table for CCD model

Source of Variation	Degree of Freedom (DF)	Sum of Square	Mean square	F-Value	P-Value
Model	9	19.4347	2.1594	40.90	<0.0001
A	1	2.0962	2.0962	39.70	<0.0001
B	1	0.8139	0.8139	15.42	0.003
C	1	0.1701	0.1701	3.22	0.052
A^2	1	16.0838	16.0838	304.64	<0.0001
B^2	1	0.8039	0.8039	15.23	0.003
C^2	1	0.2575	0.2575	4.88	0052
AB	1	0.0001	0.000	0.00	1.000
AC	1	0.0084	0.0084	0.16	0.7
BC	1	0.0072	0.0072	0.14	0.7
R^2=0.9736	19	-			

Table 5. Significance of term coefficients for CCD

Term	Coefficient	Standard Error Coefficient	T-Value	P-Value
Constant	4.1104	0.0937	43.86	0.000
pH	0.3918	0.0622	6.30	0.000
Temperature	0.2441	0.0622	3.93	0.003
Substrate conc. (%)	0.1116	0.0605	1.79	0.103
pH*pH	-1.0564	0.0605	-17.45	0.000
Temperature * Temperature	-0.2362	0.0605	-3.90	0.003
Substrate conc. * Substrate conc.	-0.1337	0.0605	-2.21	0.052
pH * Temperature	0.0000	0.0812	0.00	1.00
pH * Substrate conc.	0.0325	0.0812	0.40	0.698
Temperature* Substrate conc.	0.0300	0.0812	0.37	0. 720

7. Conclusions

Experimental investigation on ethanol production is carried out using damaged corn grains flour as substrate. SSF technique using co-culture of *A. niger* and non-starch digesting, sugar fermenting S. cerevisiae are employed. Parameters concerning Simutaneous Saccharification and Fermentation process namely, temperature, pH and substrate concentration are optimized successfully by response surface methodology. Optimized conditions for ethanol production from damaged corn grains are temperature (31^0C), pH (5.6) and substrate concentration (12%) to get maximum ethanol concentration of 4.24 (g/100 ml) by developed model. This methodology represents a valuable tool for optimization of process parameters using low cost damaged corn grains. Hence, damaged corn grains are

found to be a good substrate for ethanol production and efforts shows vast potential use of damaged or prone to be wasted food grains conversion in to fuel ethanol.

Acknowledgments

Authors are thankful to the **SSBT`s, College of Engineering and Technology, Bambhori, Jalgaon** for providing libraryfacility and **L.I.T., Nagpur, MS, India** for providing laboratory facility. Authors would like to thank the staff and colleagues for useful discussions.

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