# Simultaneous saccharification and fermentation of *Jatropha curcas* (Linn.) seed cake for production of bioethanol: a biotechnological approach

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# INTRODUCTION

- Lignocellulotic biomass is the most abundant biomass in the world which can be obtained from agricultural and other biodiversity residues like straw.
- Such lignocellulose containing biomass can be used potential source for the production of bio-fuel and solve the dependence of country on imported petroleum products

- Seedcake after exhaustive oil extraction process also reported to have significant percentage of such components (Ververis *et al.* 2007).

- Bioethanol have been produced from ligno-cellulosic biomass in a various process of hydrolysis, fermentation or dehydration of various crop biomass like sugar cane, wheat and rice straw (Mosier *et al*., 2005).

- Since, lignocellulosic materials have a complex chemical structure and high pentose fraction makes tough to hydrolysis or other process alone.

- Hence, optimized pretreatment, followed by enzymatic hydrolysis and fermentation have been suggested as a the best method (Buaban *et al.* 2010).

- In this process sugar accumulation in the system proved to be minimized and yields to be improved

- Yield have been favored by fermentation period, substrate , incubation temperature, growth conditions of the microbes. (Cazetta *et al.* 2007) ).

- However, little attention been given to the component of the Jatropha seedcake and the potential of simultaneous enzyme hydrolysis and fermentation for production of bioethanol.

- This study thus aimed at to investigate the potential use of a simple integrated process of simultaneous enzyme hydrolysis and fermentation for the production of bioethanol using Jatropha seedcake as a substrate .

- The use of appropriate statistical approach like D-Optimal to see the interaction of the independent variables and the impact on the ethanol production have been neglected.

- There is also little study so far have been done on the pretreatment of the seedcake and used for study of enzyme saccharification and fermentation for production of bioethanol

# OBJECTIVES

***General objectives***

- To investigate simultaneous saccharification and fermentation of *Jatropha curcas* (Linn.) seed cake for production of bioethanol.

***Specific objective***

- To use *Jatropha* seed cake as an alternative source for bioethanol production.
- To pretreat the *Jatropha* seedcake and increase the conversion of polymeric into monomeric carbohydrate
- To determine cellulase enzyme activity and Enzymatic saccharification
- To determine the component of the seedcake like cellulose, lignin, hemicellulose and ash.
- To optimize the condition for maximum production of bioethanol.

# METHODS AND MATERIALS

## ❖ *Sample preparation and component analysis*

- The seed of *Jatropha curcas* was procured from Suntan, Tamil Nadu, and India. Then the sample was dried and ground
- Ash, lignin, cellulose and hemicellulose contents of the seed cake were determined.
- The dried samples of 0.5gm were boiled in 5 mL of 75% w/w $H_2SO_4$ solution for 6hrs in order to hydrolyze the cellulose and hemicellulose. (W1)
- The residue was heated at $600^0$C for 6hrs and ash content (W2) was determined.
- Lignin was then calculated by the difference (W1 - W2). (Miller, 1959). Anthrone method was used to calculate total carbohydrate

## ❖ *Substrate and pretreatments*

➢The dried seedcake was pretreated with upto 2.5% NaOH and/or 4%NaOCl (Gould 1983, Ghose 1987).

➢5gm of the biomass was suspended in 50ml solution of NaOH and NaOCl in a flask at a room temperature for 4h which maintain the ratio of solid to liquid as1:10(w/v).

➢ The solid residue was collected by filtration and washed extensively with distilled water until neutral pH.

➢Pretreated biomass was dried in the oven at $60^0$C used for enzymatic hydrolysis ( Eveleigh *et al*. 2009).

## ❖ *Enzymatic saccharification*

- This experiment was carried out in stoppered conical flasks (100 ml) in the presence of 0.01% sodium azide.
- The pH was adjusted with 0.05 M citrate buffer, and cellulase was added to the pretreated substrate (2.5%, dry weight basis) in a total volume of 50ml reaction medium.
- The flasks were incubated at 50°C on an orbital shaker agitated at 150 rpm.
- Aliquots of 2 ml were taken periodically, centrifuged and the supernatants analyzed for reducing sugars. The percentage of saccharification was calculated according to (Ghose 1987).

## ❖ *Preparation of microbial inoculum*

- *Trichoderma reesei* and *Zymomonas mobilis* were obtained from NCIM (National Chemical Laboratories, Pune, India).
- To prepare the inoculum were suspended in 2ml medium and then pipetted into a 250ml Erlenmeyer flask containing 50ml of growth medium and incubated in a shaking bed (180rpm) at $30^0$C.
- The initial pH value of the medium was adjusted before being autoclaved at $121^0$C for 20min.
- After 24h grown microbes in the medium was used as the inoculum for enzymatic hydrolysis. The amount of inoculum used was 5% (v/v) of the medium in the SSF. The cultures were routinely grown as described (Lee and Huang, 1995).

## ❖ *Simultaneous saccharification and fermentation (SSF)*

- ❖ Simultaneous saccharification and fermentation was carried out in reaction mixtures consisted of treated substrate.
- ❖ The composition of the hydrolysis medium was as follows: various concentration of the pretreated biomass, enzyme loading, pH, incubation temperature and working volume.
- ❖ The SSF mixture was autoclaved and studies were carried out in 250 ml conical flasks with in various working volume.
- ❖ Thereafter culture broth was centrifuged and the supernatant was analyzed for reducing sugars and ethanol.

## ❖ *Analytical methods*

- Jatropha seed cake was analyzed for cellulose, hemicelluloses, (acid insoluble) lignin and ash (Ververis *et al.* 2007).

- Potential glucose, sugars and lignin in the substrate were determined after total hydrolysis with $H_2SO_4$ using DNS method and total carbohydrate was determined using anthrone reagent.

- Cellulase activity was assayed as FPU (filter paper units) (Eveleigh *et al*. 2009).

- Ethanol was estimated by micro-dichromate method using microplate reader (Wang *et al*. 2003; Xiao *et al*., 2004). Ethanol was used as standard (Cazetta *et al.* 2007).

## ❖ *Experimental design and Data analysis*

- The experiment was design using optimal which shows various interaction of multiple independent variable with the yield using RSM.
- The results of each D-optimal design were analyzed using Design Expert software version 7.1.5.
- All experiments were conducted in triplicate.

# RESULTS AND DISCUSSION

- ***Seedcake composition, enzyme saccharification and cellulase activity***

- The *Jatropha* seed biomass was dried over night at 50°C in an oven, ground, sieved and then taken for the study.

- Each time 50g powder were packed in soxhlet and defatted with petroleum ether (60-80°C) for 30-35 complete cycles.

- The petroleum ether extract was concentrated using Buchi-Rotavapor at reduces pressure and temperature.

- The seedcake was then made ready for further study.

- Thereafter total carbohydrate, lignin, cellulose, hemicellulose and ash contents were determined and the result is shown in figure 1

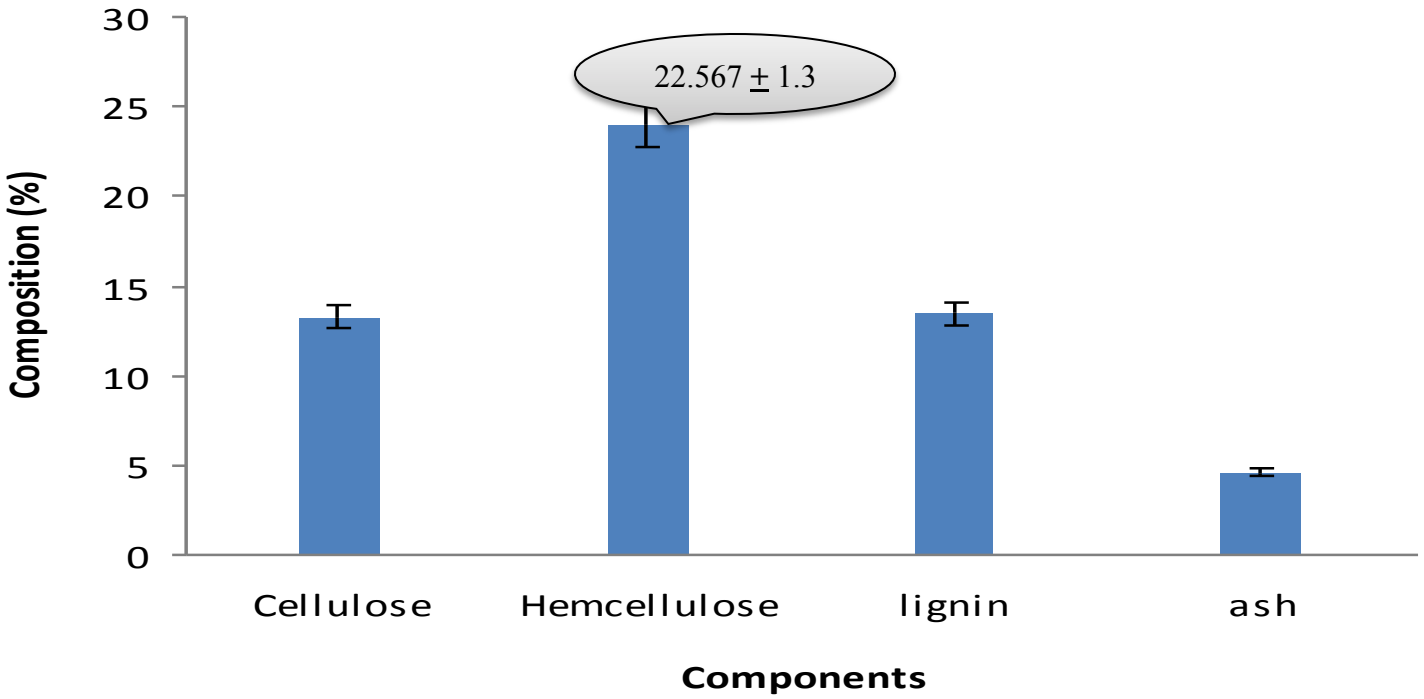


**Figure 1:** Persentage composition of lingo-celulotic material of Jatropha seedcake

- The seedcake in this study contained cellulose (13.6 ±0.95), hemicellulose (22.567 ± 1.3), and lignin (10.47±0.89) in the percentages respectively (Fig 1). The ash contain was found to be (4.6 ± 0.88)% and **total carbohydrate contain was (46.63 ± 3.07)%.**

- Cellulase activity was determined using filter paper unit and found to be 40 FPU/gm.

- Cellulase Enzyme saccharification rate was also assessed and the result is shown in figure 2.

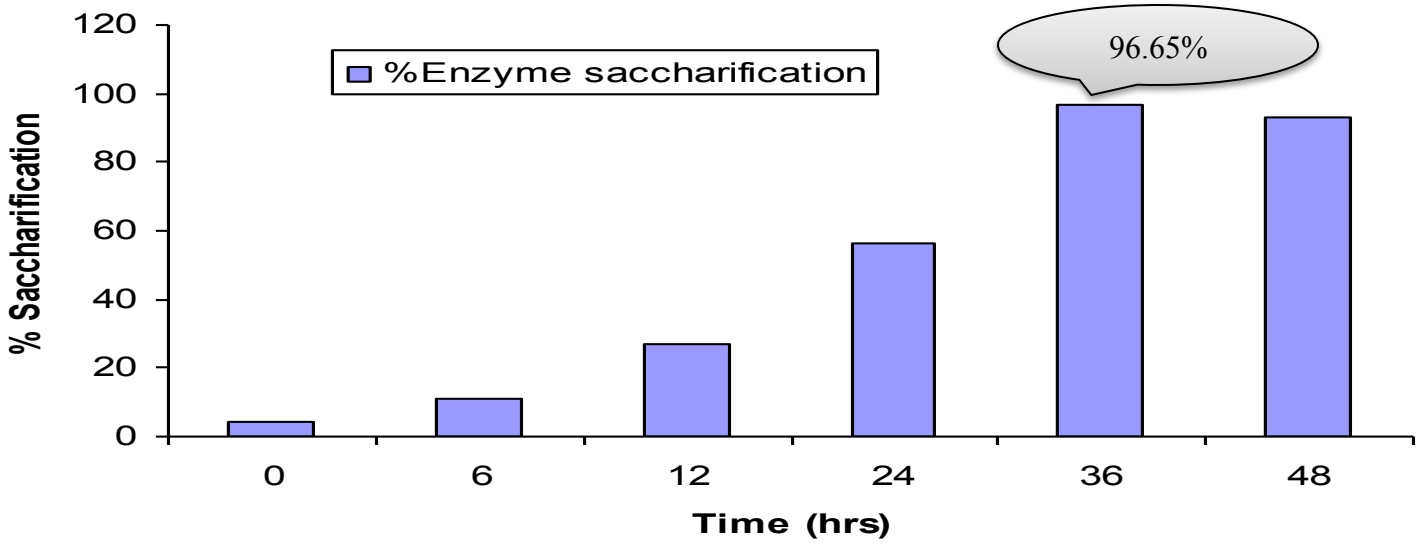


**Figure 2:** Percent of enzyme saccharification against time reaction

- The enzyme works for 96.65% conversion of the polymeric carbohydrate into monomeric carbohydrate forms at a pH=4.5 treated with NaOH at 50°C within 36hrs

## ❖ *Strain selection for Simultaneous saccharification and fermentation (SSF*

- ❖ Simultaneous saccharification and fermentation (SSF) was carried out using two microbial strains *Zymomonas mobilis* and *Trichoderma reesei* based on the report (Rogers *et al.,* 1997).
- ❖ During screening experimental factors the *T. reesei* was found to be bulky and less resistant to alcohol than *Z. mobilis*.
- ❖ Moreover, *T. reesei* has take more time of fermentation.
- ❖ Hence we used *Z. mobilis* for further optimization study.
- ❖ Among the factors selected for the cultivation of the microbes the five factors: incubation temperature, pH, enzyme load, substrate concentration and working volume were found to be superiors and considered for further optimization process.

❖ ***Optimization and validation study for production of bioethanol.***

- The experiment was performed with various parameters like pH, enzyme loading, temperature, substrate concentration and working volume using optimal RSM statistical approach.

- In the study the temperature ranges of 25-37$^0$C, pH 4-9, enzyme load ranges from 0.25-0.75%, substrate concentration from 4-8% and working volume ranged from 50ml-100ml were used.

- Hence, the Optimized condition using D-Optimal pH=4, enzyme load of 0.25%, substrate concentration of 8%, incubation temperature of 31$^0$C and working volume found to obtain **78.52% (which is some what near to the value predicted by the software)** conversion of ligno-celullotic material converted to alcohol in 24hr of incubation time (figure 4).

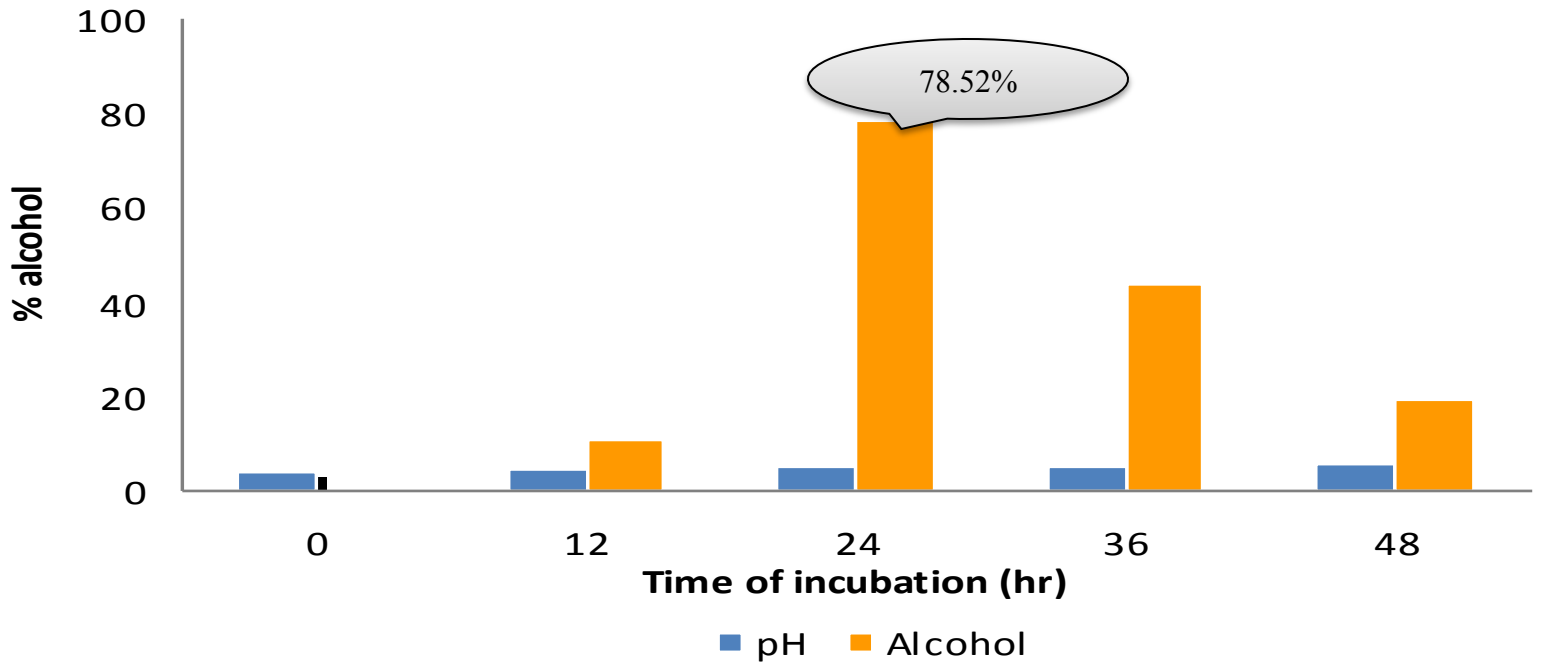


**Figure 3**: The bioethanol production and the change in pH at the optimal condition

❖ The pH of the media was found to be raised from 4 to 5.5

- In the experiment a total of 27 runs with five variables at three levels were used to optimize.

- Three-dimensional surface plots were drawn to illustrate the effects of the independent variables on the dependent variable, and described by a quadratic polynomial equation and fitted to the experimental data (Fig. 5A-D).

**The model equation**

**Ethanol (%)** =35.23+4.71 * A-3.27* B-0.76 * C+7.38* D-5.40 * E-8.21* A * B-5.26* A * C+6.82* A * D+2.5* A * E-0.27 * B * C-0.37 * B * D-9.69 * B * E+0.81* C * D-3.88* C * E-6.92* D * E+14.53 * A2 -18.95 * B2-4.24 * C2+9.14 * D2-1.76 * E2

Where the independent varioables represented as A: pH, B) enzyme load, C) Substrate concentration, D) Working volume and E) Incubation temperature

- The fit of the models was evaluated by the determination of $R^2$ coefficient =0.935.

D-optimal design experimental and predicted values of bioethanol production.

| Run | pH | Enzyme Load | Substrate concentration | Working volume | Temperature | Actual | Predicted |
|---|---|---|---|---|---|---|---|
| 1 | 4 | 0.25 | 6 | 50 | 25 | 60.22 | 61.29 |
| 2 | 4 | 0.75 | 6 | 50 | 37 | 55.83 | 55.68 |
| 3 | 6.5 | 0.75 | 8 | 100 | 25 | 27.28 | 27.33 |
| 4 | 9 | 0.75 | 8 | 50 | 31 | 12.27 | 15.36 |
| 5 | 9 | 0.25 | 8 | 75 | 25 | 10.99 | 12.39 |
| 6 | 6.5 | 0.25 | 8 | 50 | 37 | 68.09 | 70.35 |
| 7 | 4 | 0.75 | 8 | 75 | 37 | 3.30 | 0.41 |
| 8 | 4 | 0.75 | 6 | 50 | 37 | 8.79 | 5.45 |
| 9 | 4 | 0.5 | 6 | 75 | 31 | 54.91 | 51.71 |
| 10 | 4 | 0.5 | 4 | 50 | 25 | 73.40 | 71.32 |
| 11 | 7.75 | 0.63 | 7 | 62.5 | 31 | 58.02 | 55.55 |
| 12 | 9 | 0.5 | 8 | 100 | 37 | 37.34 | 32.81 |
| 13 | 6.5 | 0.25 | 4 | 100 | 25 | 24.90 | 31.23 |
| 14 | 9 | 0.25 | 4 | 50 | 25 | 49.79 | 47.54 |
| 15 | 9 | 0.25 | 4 | 50 | 37 | 36.24 | 37.69 |
| 16 | 4 | 0.5 | 8 | 50 | 25 | 19.04 | 18.37 |
| 17 | 9 | 0.25 | 4 | 100 | 37 | 16.46 | 15.13 |
| 18 | 4 | 0.25 | 4 | 50 | 37 | 73.03 | 75.47 |
| 19 | 6.5 | 0.75 | 4 | 50 | 25 | 14.10 | 9.63 |
| 20 | 9 | 0.25 | 8 | 75 | 25 | 8.79 | 8.93 |
| 21 | 6.5 | 0.75 | 4 | 100 | 37 | 15.56 | 4.30 |
| 22 | 4 | 0.75 | 4 | 100 | 25 | 24.90 | 31.56 |
| 23 | 4 | 0.25 | 6 | 100 | 37 | 51.98 | 48.12 |
| 24 | 4 | 0.25 | 6 | 50 | 25 | 15.01 | 23.52 |
| 25 | 9 | 0.75 | 4 | 100 | 25 | 36.24 | 37.11 |
| **26** | **4** | **0.25** | **8** | **100** | **31** | **78.52** | **79.58** |
| 27 | 9 | 0.75 | 4 | 50 | 37 | 6.78 | 2.13 |

ANOVA for Response Surface Quadratic Model Of D-Optimal for ethanol production

| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|
| Model | 8245.83 | 20 | 412.29 | 4.4 | 0.0371 | Significant at P<0.05 |
| A-pH | 315.53 | 1 | 315.53 | 3.36 | 0.0163 | |
| B-Enzyme load (%) | 187.44 | 1 | 187.44 | 2 | 0.0071 | |
| C-Substrate concentration (gm) | 8.32 | 1 | 8.32 | 0.089 | 0.0759 | |
| D-Working volume (ml) | 937.16 | 1 | 937.16 | 9.99 | 0.0195 | |
| E-Fermentation temperature (°C) | 516.77 | 1 | 516.77 | 5.51 | 0.0572 | |
| AB | 670.49 | 1 | 670.49 | 7.15 | 0.0368 | |
| AC | 365.25 | 1 | 365.25 | 3.9 | 0.0859 | |
| AD | 408.37 | 1 | 408.37 | 4.36 | 0.082 | |
| AE | 65.16 | 1 | 65.16 | 0.69 | 0.0664 | |
| BC | 1.07 | 1 | 1.07 | 0.011 | 0.075 | |
| BD | 2 | 1 | 2 | 0.021 | 0.087 | |
| BE | 1283.7 | 1 | 1283.7 | 13.69 | 0.0801 | |
| CD | 8.99 | 1 | 8.99 | 0.096 | 0.0673 | |
| CE | 216.35 | 1 | 216.35 | 2.31 | 0.1796 | |
| DE | 668.89 | 1 | 668.89 | 7.13 | 0.037 | |
| A2 | 787.12 | 1 | 787.12 | 8.39 | 0.0274 | |
| B2 | 564.73 | 1 | 564.73 | 6.02 | 0.0495 | |
| C2 | 49.78 | 1 | 49.78 | 0.53 | 0.4937 | |
| D2 | 126.72 | 1 | 126.72 | 1.35 | 0.2892 | |
| E2 | 4.1 | 1 | 4.1 | 0.044 | 0.8413 | |
| **Residual** | **562.62** | **6** | **93.77** | | | |
| Lack of Fit | 252.79 | 3 | 84.26 | 0.82 | 0.5644 | not significant |
| Pure Error | 309.83 | 3 | 103.28 | | | |
| Cor Total | 8808.44 | 26 | | | | |

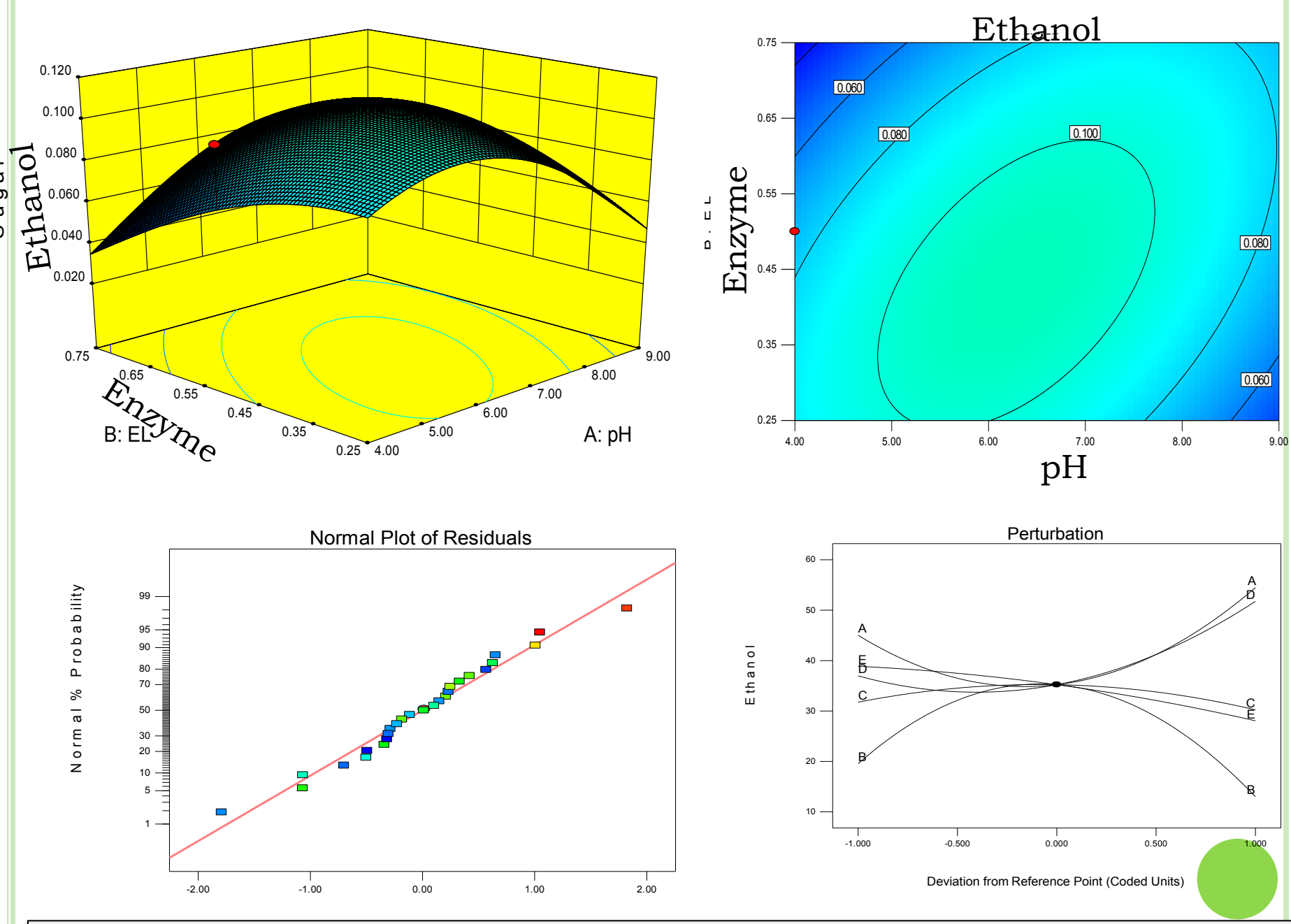


**Figure 5:** A) 3D-surface plot for production of ethanol, B) contour plot (C) Normal plot, (D) Perturbation plot for bioethanol production by *Zymomonas mobilis*

## ❖ Validation study

❖ Further validation study was under taken using the optimal condition and found to be consistent with the optimized condition (ethanol=78.47%)

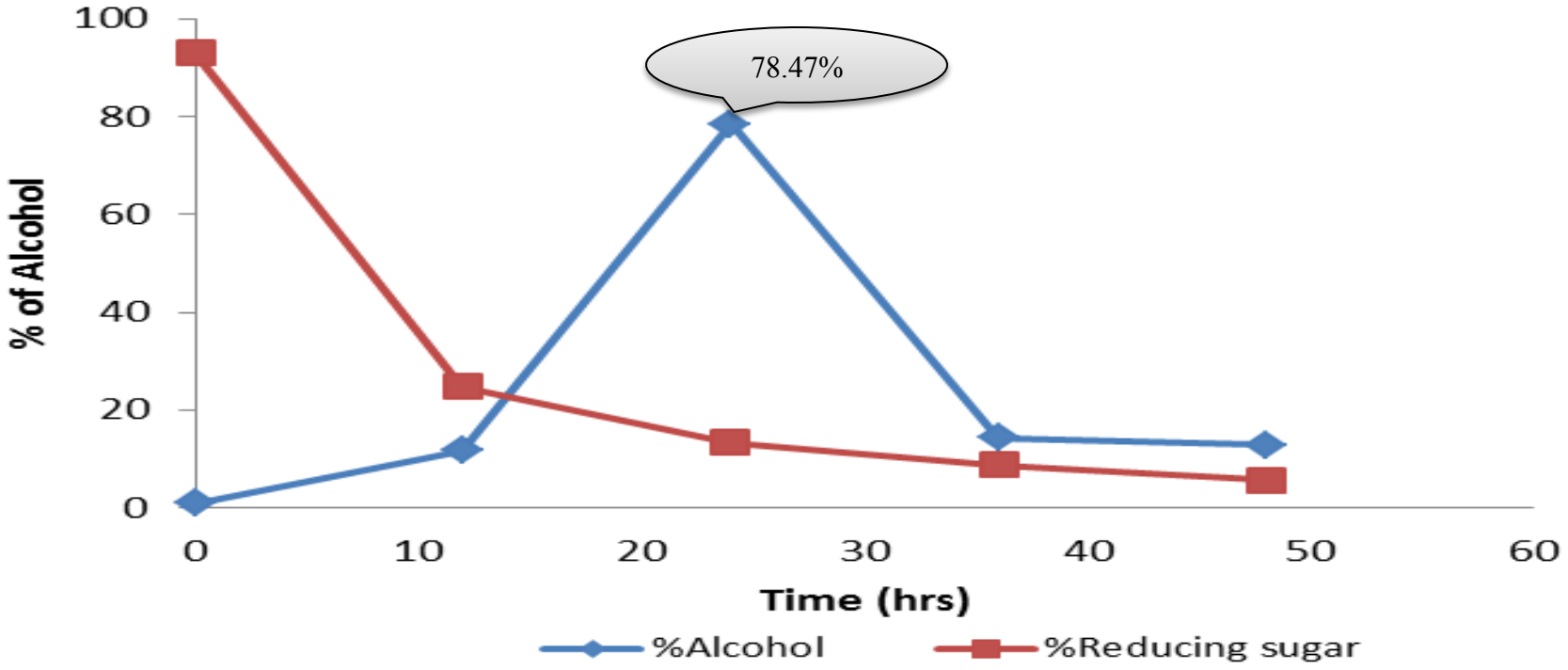


**Figure 4:.** Validation study on the production of alcohol and release of reducing sugar

# CONCLUSION AND RECOMMENDATION

❖Biotechnological conversion of lignocellulotic material provides high yield of bioethanol.

❖The results of this study showed that *Z. mobilis* is an important biological material to produce bioethanol in short time frame.

❖ pH=4, enzyme load of 0.25%, substrate concentration of 8%, incubation temperature of $31^0$C and working volume 100ml was found to be optimum condition for production of bioethanol from seedcake.

❖The accuracy of the actual experimental result (78.52 %) fitted with the predicted value of conversion of reducing sugar to ethanol in the model (**79.58%)**

❖This result can be used as a reference for further scale up studies for prodcution bioethanol at industrial level to use seedcake as a substrate.

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