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Biobutanol production from rice straw by a non acetone producing *Clostridium sporogenes* BE01

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HIGHLIGHTS

- *Clostridium sporogenes* – a novel non acetone forming butanol producer isolated.
- Biobutanol produced using a lignocellulosic biomass hydrolysate derived medium.
- Anionic resin successfully removed fermentation inhibitors from hydrolysate.
- A maximum butanol production of 5.52 g/l in rice straw hydrolysate attained.
- One of the highest butanol production by *C. sporogenes* reported in any medium.

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ABSTRACT

Biobutanol from lignocellulosic biomass has gained much attention due to several advantages over bioethanol. Though microbial production of butanol through ABE fermentation is an established technology, the use of lignocellulosic biomass as feedstock presents several challenges. In the present study, biobutanol production from enzymatic hydrolysate of acid pretreated rice straw was evaluated using *Clostridium sporogenes* BE01. This strain gave a butanol yield of 3.43 g/l and a total solvent yield of 5.32 g/l in rice straw hydrolysate supplemented with calcium carbonate and yeast extract. Hydrolysate was analyzed for the level of inhibitors such as acetic acid, formic acid and furfurals which affect the growth of the organism and in turn ABE fermentation. Methods for preconditioning the hydrolysate to remove toxic end products were done so as to improve the fermentation efficiency. Conditions of ABE fermentation were fine tuned resulting in an enhanced biobutanol reaching 5.52 g/l.

1. Introduction

Biobutanol is gaining attention as a liquid transportation fuel because of its similarity in properties to gasoline besides being renewable (Ranjan et al., 2012). Its high energy content, lower affinity to water and better blending capacities make it more desirable than ethanol. Butanol can be produced both petro chemically and fermentatively (Durre, 2007). Butanol production from sugars by using *Clostridium* species is an industrially established process. The challenge of ABE fermentation is its low productivity, high cost of the substrate, product toxicity and its separation from fermentation broth. In order to overcome problems like substrate cost and availability, research is being focused on renewable agriculture residues as feed stock for biobutanol production (Qureshi et al., 2008). Rice straw is one of the most abundant lignocellulosic biomass available. It has high cellulose and hemicellulose content which can be readily hydrolyzed into hexoses and pentoses (Binod et al., 2010). Hexoses are readily utilized by solventogenic *Clostridium* species, Pentoses can also be utilized, but with low production rates (Volesky and Szczesny, 1983).

In Acetone, Butanol Ethanol (ABE) fermentation with known solventogenic Clostridial species, acetone is also produced in addition to butanol and ethanol. Acetone does not qualify as fuel and should be separated from the final products which results in net low yield of solvents (Kannan et al., 2010). Solventogenic *Clostridium sporogenes* can ferment sugars to organic acids like acetic acid and butyric acid and alcohols such as ethanol and butanol, by producing gaseous byproducts like carbon dioxide and hydrogen (Turton et al., 1983; Leja et al., 2011). *C. sporogenes* is a non neurotoxigenic counterpart of group 1 *Clostridium botulinum* (Sebaihia et al., 2007) and it produces ethanol and butanol without acetone in the final mixture of solvents, which is an advantage in bioconversion process of biomass to alcoholic fuels (Kannan et al., 2010).

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The present study evaluated the production of butanol production using an enzymatic hydrolysate of dilute acid pretreated rice straw and optimized the parameters for improving yield of butanol using *C. sporogenes* BE01.

2. Methods

2.1. Rice straw pretreatment and hydrolysis

Rice straw obtained locally was knife milled to a powder with maximum particle size of ~4 mm and its composition analysis was done to estimate cellulose, hemicellulose, lignin and other components (Sluiter et al., 2004, 2005). Rice straw was pretreated with 4% (w/w) H_2SO_4 at a solid loading of 15% (w/w) and at 121 °C for 60 min. Solid mass was separated from the pentose containing liquid stream and was air dried at room temperature to remove excess moisture. The final moisture content of the biomass was determined and the pretreated straw was used for enzymatic hydrolysis.

Enzymatic hydrolysis was done at 10% (w/w) solids loading. Commercial acid cellulase (Zytex India Limited, Mumbai) was used for saccharification of the pretreated rice straw. Enzyme was used at 30 FPUs/g (dry substrate) concentration and the hydrolysis was performed at 50 °C and 200 rpm for 48 h. Citrate buffer (0.05 M) was used as buffering agent to maintain the pH 4.8. The hexose stream obtained from hydrolyzed rice straw was separated from the un-hydrolyzed biomass by centrifugation and was used for fermentation.

2.2. Micro organism and culture conditions

C. sporogenes BE01 was isolated from contaminated cooked meat medium at the Biotechnology division of CSIR–NIIST. The culture was maintained as a spore suspension at 4 °C. It was cultivated in Tryptone/glucose/yeast extract (TGY) medium to generate the pre-inoculum and inoculum for butanol fermentation. TGY medium contained in g/l Tryptone – 5, Yeast extract – 5, K_2HPO_4 – 1 and Glucose – 1. Cysteine HCl, 0.5 g/l was added as chemical reducing agent and resazurin, 0.01% (w/v) was added as redox indicator in the medium (Fukushima et al., 2002). To prepare pre-inoculum, spores were heat shocked at 75 °C for 2 min and kept in ice to bring it to room temperature. Heat shocked spores (10% v/v) were inoculated into TGY medium and incubated at 37 °C for 12 h in an anaerobic chamber (Bactron I, Shelllabs, USA) purged with nitrogen gas. Actively growing cells from pre-inoculum were inoculated into fresh TGY medium to generate inoculum for fermentation. Growth of the organism was measured by taking optical density at 540 nm.

2.3. Detoxification of hydrolysate

Enzymatic hydrolysate of rice straw was filtered through glass microfiber filters to remove fine suspended particles. Amberlite resins XAD4, XAD7, XAD16 (Dow, USA) and the anionic resin Seralite SRA400 (SRL, India) were screened to remove inhibitors from rice straw hydrolysate. Properties of the resins are given in Table 1. Inhibitors such as acetic acid, formic acid, furfurals and hydroxymethyl furfurals present in hydrolysate were analyzed prior to detoxification and the detoxification efficiency was determined by analyzing the remaining acids and furfurals present in resin treated samples. Detoxification studies were performed in batch process. Before using the resins for detoxification studies, they were prepared as recommended by the supplier. Resins were added to the hydrolysate at 1% (w/v) and kept at 200 rpm for 8 h at 30 ± 2 °C. Selection of the resin for detoxification was done based on the fermentation performance of the hydrolysates detoxified using each resin. For further detoxification studies, 60 g of selected resin was packed in the column and the clear hydrolysate was passed through the resin at a flow rate of 8 ml/min.

2.4. Fermentation

Fermentation was performed with rice straw hydrolysate as the medium in 100 ml screw capped bottles containing 50 ml medium. The medium was supplemented with minerals [in g/l $(NH_4)_2SO_4$ – 1.5, $MgSO_4{\cdot}7H_2O$ – 6, KH_2PO_4 – 0.5, NaCl – 0.01, $MnSO_4{\cdot}H_2O$ – 0.01, $FeSO_4{\cdot}7H_2O$, 0.01] (Hartmanis et al., 1986; Qureshi and Blaschek, 1999) or without. Yeast extract (1.5 g/l) was added as the source of organic nitrogen, vitamins and other essential nutrients. Calcium carbonate (4.5 g/l unless specified different) was added as buffering agent in the hydrolysate and the medium pH was adjusted to 6.7. The fermentation medium was sterilized by autoclaving (121 °C for 15 min) and cooled down to 37 °C under continuous purging of nitrogen gas. Actively growing 12 h old culture of *C. sporogenes* BE01 at 10% v/v was used as inoculum for butanol production, unless age and percentage of the inoculum is specified different.

2.5. Effect of inoculum age on butanol production by C. sporogenes BE01 in batch fermentation

Clostridium sporogenes BE01 was used as the inoculum for butanol fermentation in its active growth stage. Initial pH of the fermentation medium was maintained at 6.7. Cells grown in TGY medium were harvested at 6, 9, 12 or 24 h of growth and were used as inoculum to study the impact of inoculum age on butanol production. Inoculum concentration (10% v/v) was kept constant and fermentation was continued till 96 h. Samples were collected at regular time intervals and were analyzed for butanol production.

2.6. Effect of calcium carbonate on butanol production

Calcium carbonate was used as the buffering agent to maintain the pH of the medium during fermentation (Richmond et al., 2011). The effect of calcium carbonate on accumulation of butanol in the fermentation medium was studied by adding different concentrations 0, 2, 5 and 10 (g/l) in the fermentation medium. pH was adjusted to 6.7 after adding calcium carbonate into the hydrolysate and the medium was autoclaved at 121 °C for 15 min. Fermentation was performed for 96 h and samples were collected at regular time intervals to analyze butanol production.

2.7. Optimization of butanol production

Central composite design was used to study the effect of independent variables and their interactions on the response (Butanol yield). The three important parameters studied were pH, inoculum concentration and calcium carbonate concentration. Minitab 15 (Minitab Inc., USA) was used for designing the experiment and also for data analysis and quadratic model construction. Response surface graphs were generated by the software to understand the effect of significant variables and their interactions. The optimum levels of these individual parameters and their combinations were derived from the graphs. For the three variables, the second order polynomial model equation fitted was:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where Y is the response, β_0, β_1, β_2 and β_3 are regression coefficients and X_1, X_2 and X_3 were coded values of pH, inoculum concentration and $CaCO_3$ concentration, respectively.

Table 1
Properties of adsorbents.

Properties	XAD4	XAD7	XAD16	Seralite 400
Matrix and active group	Styrene–divinylbenzene	Acrylic ester	Polystyrene, a polar	Styrene/divenyl benzene with quarternary ammonium functional group
Particle size	20–60 mesh	20–60 mesh	20–60 mesh	20–50 mesh
Surface area	725 m^2/g	~450 m^2/g	800 m^2/g	–
Pore size	~0.98 ml/g pore volume 40 Å mean pore size	1.14 ml/g pore volume	1.82 ml/g pore volume100 Å mean pore size	–
Ionic form and Ion exchange capacity	–	–	–	Cl^- 3–3.5 meq/g

2.8. Analyses

Butanol, ethanol and volatile acids produced during fermentation were analyzed by gas chromatograph (Chemito GC 8610). Poropak Q® column was used for seperation of butanol, ethanol, butyric acid and acetic acid and the separated components were detected by flame ionization detector (FID). Injector and detector temperature were 150 and 250 °C, respectively, and oven temperature was maintained as a gradient with rise in temperature from 50 to 200 °C at the rate of 8 °C/min.

Sugars were analyzed and quantified by Shimadzu Prominence UFLC with RI detector. Rezex® RPM monosaccharide analysis column (Phenomenex) was used for the analysis of sugars at oven temperature 85 °C. De-ionized water at a flow rate of 8 ml/min was used as mobile phase and each sample was run for 30 min. Rezex® ROA organic acid analysis column (Phenomenex) at oven temperature 50 °C was used for inhibitor analyses. PDA detector was used for detection and the analysis time was 50 min. Mobile phase used for separation was 0.05 M H_2SO_4 at the flow rate 0.6 ml/min.

3. Results and discussion

3.1. Comparison of biobutanol production in rice straw hydrolysates with and without mineral supplementation

Native rice straw had a composition of 47.57% cellulose, 15.75% hemicellulose, 8.66% lignin, 17.16% extractives and 9.75% ash. Hydrolysate with 39.02 g/l of glucose, 11.35 g/l xylose and 1.71 g/l arabinose was obtained with dilute acid pretreatment and enzymatic hydrolysis of rice straw. Hemicellulose is removed with dilute acid pretreatment and enhances the enzymatic digestibility of cellulose from residual solids (Mosier et al., 2005). Butanol fermentation using rice straw hydrolysate was tried both with supplementation of minerals or without. With supplementation of minerals in hydrolysate, 2.79 g/l of butanol, 1.62 g/l of ethanol, 1.21 g/l of acetic acid and 3.16 g/l of butyric acid were produced and without mineral supplementation, 3.43 g/l and 1.89 g/l of butanol and ethanol, respectively, were produced including 1.0 g/l acetic acid and 2.96 g/l butyric acid (Fig. 1a). Hexoses and pentoses were utilized by the organism for butanol production. forty four percent of glucose, 56% of xylose and 59% of arabinose was utilized by the organism from the hydrolysate without mineral supplementation and 48% of glucose, 54% of xylose and 69% of arabinose was utilized by the organism from the hydrolysate with mineral supplementation (Fig. 1b). Solvent production was higher without mineral supplementation; this could be due to the natural presence of minerals in rice straw like potassium, phosphorous, iron and calcium in their oxidized forms and also small amounts of magnesium, sulfur and sodium (Kadam et al., 2000). Mussatto and Roberto (2005) reported decrease in xylitol production with addition of nutrients to rice straw hydrolysate medium and also

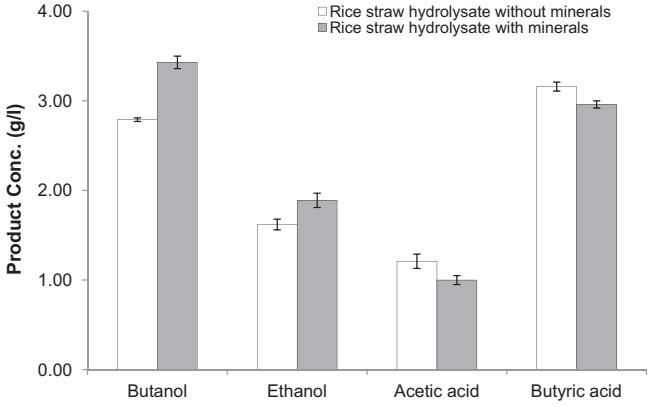

Fig. 1a. Comparison of acids and solvents production by *C. sporogenes* BE01 in rice straw hydrolysate with and without mineral supplementation.

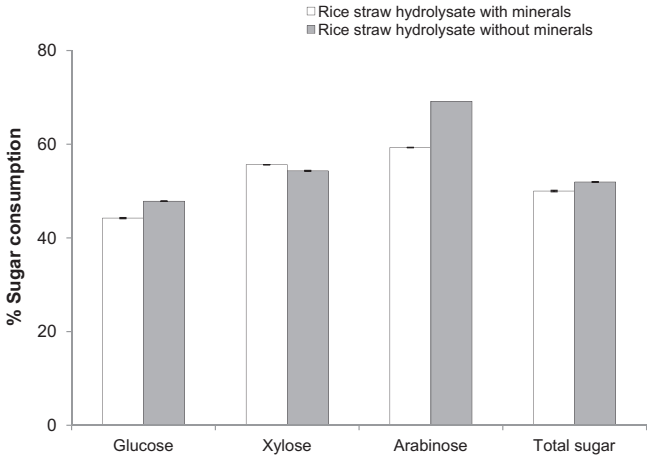

Fig. 1b. Comparison of sugar utilization by *C. sporogenes* BE01 from rice straw hydrolysate with and without mineral supplementation.

mentioned that it could be probably due to imbalance between ionic nutrition. Ammonium sulfate can also have negative effect on the organism and fermentation by releasing sulfate ions and decreasing the pH of the medium (Mussatto and Roberto, 2005).

3.2. Effect of inoculum age on butanol fermentation

The results shown in Fig. 2 indicated that the age of inoculum influences solvent production. The 12 h old inoculum supported higher butanol production (3.32 g/l) when compared to other inocula. There was a decrease in butanol production when 24 h old inoculum was used. Inoculum age and the culture motility control the production of solvents and trigger the activity of enzymes

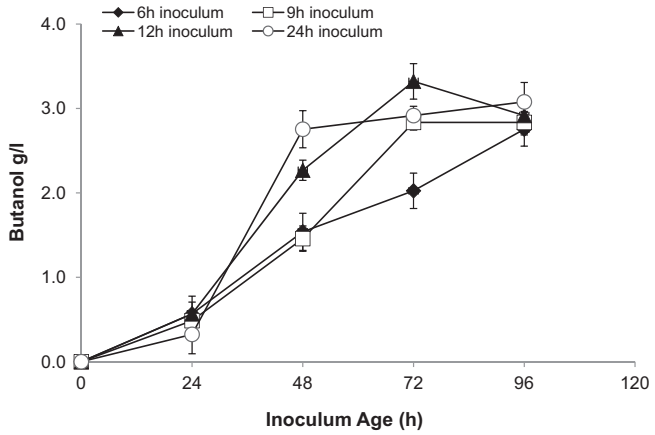

Fig. 2. Effect of inoculum age on butanol production.

operating in solventogenic phase (Spivey, 1978). Jones et al. (1982) correlated the morphological changes of *Clostridium acetobutylicum* P262 with its solvent production in molasses medium. It was reported that after 6 h, the chains formed by elongated rods broke and highly motile cells were released and after 14–18 h the motility of the cells decreased. Decrease in motility of the cells results in decrease in solvent production (Spivey, 1978), which explains the higher butanol yield with inoculum grown for 12 h when the cells are actively growing and motile.

3.3. Effect of calcium carbonate on butanol production

Hydrolysate supplemented with 10 g/l calcium carbonate supported the highest production of butanol (4.05 g/l) with a productivity 0.04 g l^{-1} h^{-1}. Final butanol concentration with 0, 2, and 5 g/l of calcium carbonate were 3.32, 2.35 and 1.13 g/l, respectively (Fig. 3). ABE fermentation with poorly buffered medium leads to excessive accumulation of acids. Un-dissociated acetic acid and butyric acid are inhibitory to cell growth, nutrient uptake and butanol production (Ezeji et al., 2005). Richmond et al. (2011) reported that calcium carbonate favored growth and butanol production of *Clostridium* species. It also increases the tolerance of organism to butanol and so increases the final butanol accumulation in the fermentation medium

3.4. Solvent production in detoxified rice straw hydrolysate

Dilute acid pretreatment of lignocellulosic biomass releases some compounds like acetic acid, formic acid, furfurals and hydroxy methyl furfurals which are inhibitory for conversion of sugars to desired products (Wickramasinghe and Grzenia, 2008). Removal of inhibitors is considered necessary to improve the solvent production by the organism. XAD7, XAD4, XAD16 and Seralite SRA-400 were evaluated for removal of inhibitors. Furfurals were found to be absent in hydrolysate, so removal of acetic acid and formic acid was studied. XAD7 and Seralite SRA-400 were found to be efficient in acid removal, where as XAD16 and XAD4 were less effective (Table 2). Butanol fermentation was also performed with the detoxified samples. Hydrolysate treated with Seralite SR-400 supported fermentation most efficiently compared to the other resins, attaining a butanol production of 4.78 g/l. Hydrolysates treated with XAD4 did not support improved production of solvents, but those treated with XAD16 and XAD7 supported higher the butanol production reaching 4.13 and 4.29 g/l, respectively. XAD4 treated hydrolysates gave similar butanol production (3.20 g/l) when compared to non detoxified hydrolysate, which gave 3.3 g/l. Total solvent yield was also higher in hydrolysate treated with Seralite – SRA400 (7.78 g/l) (Fig. 4). Enzymatic hydrolysate did not show any presence of furfurals and hydroxymethyl furfurals. Furfurals and hydroxymethyl furfurals which are generally formed by acid hydrolysis can be removed efficiently by XAD4 resin (Qureshi et al., 2008). Anionic exchanger can efficiently remove both anionic and uncharged inhibitors at higher pH 10.0 (Nilvebrant et al., 2001). They can also efficiently remove acids like levulinic acid, acetic acid, formic acid and furfurals (Chandel et al., 2011) which explain the better performance of Seralite SRA400 for detoxifying the hydrolysate.

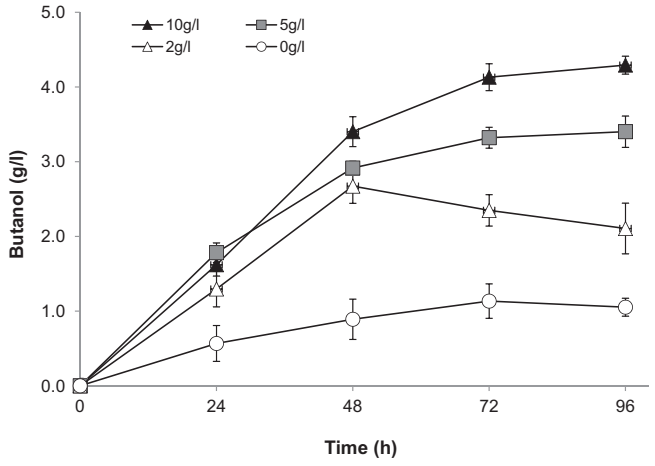

Fig. 3. Effect of $CaCO_3$ concentration on butanol using rice straw hydrolysate.

3.5. Time course study of butanol production with detoxified and non detoxified hydrolysates

Anionic resin (Seralite SRA400 was found better for detoxification of rice straw hydrolysate and hence used for further detoxification studies. The maximum butanol production (4.62 g/l) was obtained with detoxified hydrolysate at 96 h with a productivity of 0.05 g l^{-1} h^{-1}. Non detoxified hydrolysate also supported a maximum butanol production of 3.32 g/l at 96 h with a productivity of 0.03 g l^{-1} h^{-1}. Butanol production in detoxified hydrolysate increased from 72 to 96 h, compared to the non-detoxified hydrolysate in which the butanol production had dropped on extended incubation from 72 to 96 h (Fig. 5). Ranjan et al. (2012) had reported an butanol production of 12.7 g/l and a yield of 0.38 g^{-1} which was achieved with enzymatically hydrolyzed acid pretreated rice straw by using *C. acetobutylicum* MTCC 481. In an earlier report, rice straw and sugarcane bagasse hydrolysates were detoxified with ammonium sulfate precipitation and activated charcoal treatment and ABE concentration of 18.1 g/l was obtained (Soni et al., 1982). Qureshi et al. (2008) reported that dilute acid treated corn fiber hydrolysate inhibited the cell growth and butanol production and detoxification with XAD4 resin is needed to improve the fermentation efficiency.

3.6. Response surface optimization of butanol production in rice straw hydrolysate

The study of parameters optimization by central composite design for butanol production gave the following polynomial equation for the model.

$$\begin{aligned}\text{Butanol (g/l)} = {} & -64.575 - 0.2673X_1 + 0.2079X_2 + 21.3261X_3 \\ & - 0.03101X_1^2 - 0.03013X_2^2 - 1.666X_3^2 \\ & + 0.02458X_1X_2 + 0.09843X_1X_3 - 0.0229X_2X_3.\end{aligned}$$

X_1, X_2 and X_3 are inoculum age, pH and calcium carbonate, respectively. pH and inoculum age were found to be significant variables

Table 2
Screening of resins for removal of acetic acid and formic acid from rice straw hydrolysate.

Resin	Acetic acid (g/l)			Formic acid (g/l)			Removal of total acids (%)
	Initial	Final	Removal (%)	Initial	Final	Removal (%)	
XAD4	3.46	1.51	56.36	7.24	4.16	42.54	47.01
XAD7	3.46	0.94	72.83	7.24	3.21	55.66	61.21
XAD16	3.46	1.95	43.64	7.24	2.5	65.47	58.41
Seralite SRA-400	3.46	1.06	69.36	7.24	3.28	54.70	59.44

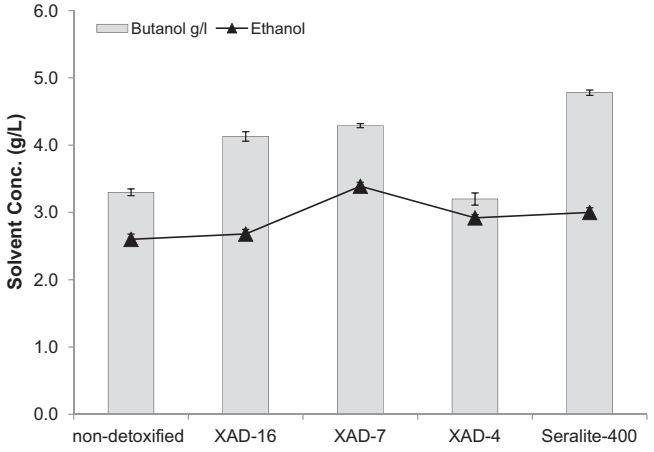

Fig. 4. Comparison of the resins used for hydrolysate detoxification.

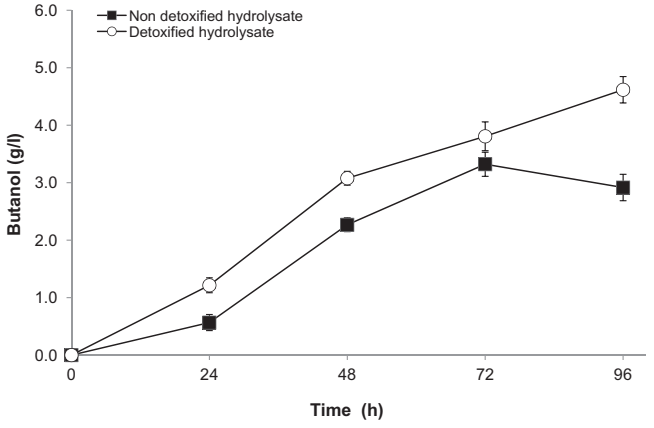

Fig. 5. Butanol production in detoxified and non-detoxified hydrolysates.

with *P* values 0.038 and 0.02, respectively. Design was found to be significant with lack of fit >0.05 and R^2 – 79.20%. Maximum butanol concentration, 5.52 g/l was obtained with 8% (v/v) inoculum, 3 g/l calcium carbonate and pH 6.8. This is comparable to the solvent production (6.63 g/l) reported by Qureshi et al. (2008) in corn fiber hydrolysate by *Clostridium beijerinki* BA101 with supplementation of minerals and vitamins.

Experimental design matrix and the responses are shown in Table 3. The response surface plot showed interaction between pH and inoculum age (Fig. 6). Maximum response was found with inoculum range 7.5% (v/v) to 8.5% (v/v) and at pH range 6.5–6.7, which is near neutral. Near neutral pH minimizes the chance of acid crash and calcium carbonate contributes for buffering of the medium. Previous study conducted by Ezeji et al., 2005 with *C. beijerinki* BA101 where fermentation was initiated at near neutral pH 6.8 and then allowed to drop to 5 or 5.5 for solventogenesis showed that the culture was able to maintain pH throughout the fermentation without requirement for external pH adjustment.

Table 3
Central composite design matrix for optimization of butanol production.

Run order	Pt type	Blocks	Inoculum (% v/v)	$CaCO_3$ (g/l)	pH	Butanol (g/l)
1	−1	2	6	6.9	6.4	4.41
2	−1	2	6	2.0	6.4	4.91
3	−1	2	6	4.5	5.7	4.03
4	−1	2	2.7	4.5	6.4	4.33
5	0	2	6	4.5	6.4	5.39
6	−1	2	6	4.5	7.0	4.23
7	−1	2	9.2	4.5	6.4	4.69
8	0	2	6	4.5	6.4	5.02
9	1	1	4	3	6.8	5.05
10	0	1	6	4.5	6.4	4.8
11	0	1	6	4.5	6.4	5.05
12	1	1	4	6	6	4.13
13	1	1	4	3	6	4.54
14	1	1	8	6	6.8	5.35
15	1	1	8	3	6	4.58
16	1	1	4	6	6.8	4.7
17	0	1	6	4.5	6.4	5.22
18	1	1	8	6	6	4.83
19	0	1	6	4.5	6.4	5.01
20	1	1	8	3	6.8	5.52

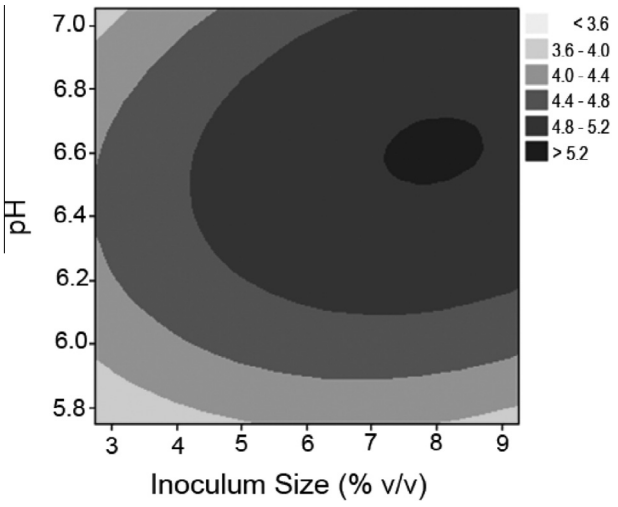

Fig. 6. Contour Plot showing interaction effects of pH and inoculum size on of butanol production.

4. Conclusion

Detoxified rice straw hydrolysate supplemented with yeast extract and calcium carbonate supported a butanol yield of 4.46 g/l and a productivity of 0.05 g l^{-1} h^{-1} using *Clostridium sporogenes*. Though the culture is not considered as an efficient producer of butanol in comparison with commercial strains like *C. acetobutylicum*, it produced 5.52 g/l of butanol under optimized conditions which is one of the highest reported for the organism on any

substrate. The ability of the culture to grow and produce butanol in rice straw hydrolysate indicates the potential to be used for bio-butanol production.

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