J Biochem Tech (2016) 7(2): 1063-1068
ISSN: 0974-2328

# Optimization for butanol production using Plackett-Burman Design coupled with Central Composite Design by *Clostridium beijerenckii* strain CHTa isolated from distillery waste manure

**Kajal G Singh, Kaushal L Lapsiya, Rekha R Gophane, Dilip R Ranade***

Received: 16 October 2015 / Received in revised form: 05 August 2016, Accepted: 18 September 2016,
Published online: 20 September 2016, 

## Abstract

Plackett-Burman and Response Surface Methodology were used to optimize fermentation medium for enhancing butanol production by *Clostridium beijerenckii* strain CHTa. Optimization using Plackett-Burman design showed yeast extract, $MgSO_4.7H_2O$ and glucose to have significant impact over other factors, namely $(NH4)_2SO_4$, $CaCO_3$, $FeSO_4.7H_2O$, cysteine hydrochloride, sodium thioglycolate and pH. To find out the optimal concentrations and interactions between the selected factors, further experiments were carried by applying Central Composite Design. The experimental results demonstrated glucose and $MgSO_4.7H_2O$ as the key components over yeast extract responsible for production of butanol by strain CHTa. A mathematical model was established to predict the optimum values for factors. Under optimal conditions, practical butanol yield of 10.08 g $L^{-1}$ and productivity of 0.45 g $L^{-1}$ $h^{-1}$ were obtained.

**Keywords:** Butanol fermentation, Plackett-Burman design, Response surface methodology, Central composite design

## Introduction

Increasing environmental concerns and changing crude oil prices have led to the attention being diverted towards developing economical and environment friendly energy resources. Properties like high energy content, low volatility, less hygroscopicity and less corrosive nature makes butanol a superior fuel than ethanol (Tashiro and Sonomoto 2010). With the increasing use of alternative fuels, butanol is once again finding its place as an alternative renewable fuel with a demand of over one billion litres per year (http://www.butalco.com/biofuels.html). Butanol has many high-value applications in manufacture of fuel, paint, adhesives, cleaners and personal care products, as well as many specialty applications.

Currently, the petroleum based butanol market has crossed $5 billion per year, with an annual growth rate of approximately 4% (http://www.cobalttech.com). Bacteria belonging to the genus *Clostridium* are reported to produce butanol from a large range of carbohydrates via Acetone Butanol Ethanol (ABE) fermentation (Lopez-Contreras et al. 2003). Under normal growth conditions, *Clostridium* has a low yield of butanol per gram of glucose. The economic viability of any fermentation is governed mainly by the raw material cost, the high product titres and solvent recovery costs. Many strategies have been implemented to obtain higher yields of butanol. One of these is manipulation of the metabolic networks within bacterial cell to prioritize the synthesis of butanol via metabolic engineering and/ or by genetic engineering (Tashiro et al. 2013). Simpler and cost effective strategies such as optimization of medium components and physiological factors can also be used to increase the yield of butanol without adding much to the manufacturing cost (Bas and Boyac 2007).

Several media have been reported to produce butanol from different *Clostridia* spp (Fouad et al. 1975). Important variables like carbon, nitrogen sources, inorganic salts and growth factors affect the growth of bacteria. ABE fermentation is typical biphasic fermentation where drop in pH due to growth triggers butanol production which makes it as a pH dependent process (Tashiro et al. 2004). So along with nutritional factors, pH also needs to be optimized.

Medium optimization is either done by empiric or statistical methods. The traditional "one factor at a time approach" is not only time consuming but also leads to an incomplete understanding of the system behaviour. It has an incapability to reach the actual optimum level. It assumes the process response as a direct function of the single varied parameter without taking into consideration the interactions of various growth parameters. In contrast, the interactive influences of the different parameters lead to the observed behaviour of growth (He et al. 2004). Response surface methodology (RSM) is a group of mathematical and statistical techniques for empirical model building that can be used to carefully design the experiments and optimize a response which is influenced by several independent variables (Bezerra et al. 2008). It can also be used to study relationships between one or more responses and a number of independent variables of growth and product

**Kajal G Singh, Kaushal L Lapsiya, Rekha R Gophane, Dilip R. Ranade***

Bioenergy Group, MACS Agharkar Research Institute, G.G. Agarkar Road, Pune 411004, Maharashtra, India

* Tel.: +91-20-25325115, E-mail: drranade@gmail.com

yield. The methodology generates a mathematical model that accurately describes the overall process. In our effort to develop a bioprocess for butanol production, a butanol producing *Clostridium beijerinckii* strain CHTa was isolated. In the present study, optimization of nutritional parameters and pH in fermentation media was investigated to improve the yield of butanol from glucose in a batch process by RSM.

## Materials and Methods

*Bacterial strain, chemical reagents and culture conditions*

In the research program on butanol fermentation, 207 isolates of strict anaerobes were isolated from various sources like digestate of distillery waste, market waste, compost samples, rumen fluid, estuary sediment, mangrove sediments and fecal matter of zoo animals. The bacterial strain CHTa used in the present study was isolated from manure of anaerobically digested distillery waste. The strain CHTa (GenBank accession number KJ169574.1) showed over 98% 16S rRNA gene sequence identity with *Clostridium beijerinckii* strain AAU1. The basal medium used for cultivation of the strain contained (g $L^{-1}$) glucose, 10; yeast Extract, 3; peptone, 10; sodium acetate, 3; sodium chloride, 5; cysteine hydrochloride, 0.5; and sodium thioglycolate, 1 (Cheng et al. 2012). Production medium as described by Cheng et al. (2012) was used for butanol production. It contained (g $l^{-1}$) glucose, 60; $(NH_4)_2SO_4$, 2; $K_2HPO_4$, 2; $CaCO_3$, 3; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.516; yeast extract, 5.1330; cysteine hydrochloride, 0.5; and sodium thioglycolate, 1. All incubations were carried out at 37°C by following anaerobic techniques based on Hungate (1960) and its modifications done by Miller and Wolin (1974) as described by Ranade and Gadre (1988).

*Batch fermentation*

All the anaerobic batch fermentations were carried out at 37°C in 130 ml serum bottles with 50 ml working volume. Samples of the fermentation broth were taken after every 24 h. All experiments were carried out in triplicates.

*Estimation of solvents and residual sugars*

Samples were centrifuged at 10,000 rpm for 10 min and the cell free supernatant was then used for analysis. Glucose was estimated by DNSA methods as described by Miller (1959). Fermentation products were analyzed on the gas chromatograph (Bruker 450- GC) equipped with flame ionization detector and 30 m×0.32 mm inner diameter capillary column (Stabilwax®-DA Columns (fused silica), Crossbond® Carbowax® polyethylene glycol). The temperatures of the oven, injector and detector temperatures were 80°C, 150°C and 200°C. Nitrogen was used as the carrier gas.

*Statistical analysis*

Determination of the most important variable (s) of the medium for butanol production is carried out before understanding the relationship between them. This was carried out in relatively few experiments using factorial designs like Plackett-Burman design (P-B) followed by Central Composite Design (CCD).

*Plackett-Burman design (P-B)*

The P-B design was used to select significantly influencing variable (s) for butanol production. Twelve runs of P-B design matrix was generated using statistical software package Design Expert 6.0 that created the design and then their effects (Table 1 and 2) were studied on butanol production.

*Central Composite Design (CCD)*

RSM was used to optimize the factors which were selected through P-B design based on CCD. This design can be helpful to investigate linear, second order, and cross-product effects of the reaction condition variables on the butanol production (Lin et al. 2011). The polynomial equation obtained after regression analyses corresponding to the stationary point and the changing of the other two variables, was expressed as three dimensional response surfaces and contour plots to illustrate the relationship between the responses and interaction effects of the variables. The design matrix was performed using Design Expert 6.0. The significant factors identified in P-B experiment were employed in CCD.

*Analysis of Variance (ANOVA)*

The determination of the statistical significance of the model terms affecting the response (butanol production) was then done by performing ANOVA. It was performed using Design Expert 6.0.

*Validation of results*

To validate and confirm the optimization, experiments were conducted in triplicates under the optimized conditions obtained after P-B and CCD. This validated the effectiveness of the RSM approach for optimizing the operational conditions for the butanol production.

Table 1: Range of values for P-B design

| | | | Actual values (g $L^{-1}$) | |
|---|---|---|---|---|
| Factor | Chemical component | Coded values | low actual | high actual |
| A | $(NH_4)_2SO_4$ | -1,0,1 | 1 | 3 |
| B | $K_2HPO_4$ | -1,0,1 | 1 | 3 |
| C | $CaCO_3$ | -1,0,1 | 2 | 4 |
| D | $MgSO_4.7H_2O$ | -1,0,1 | 0.25 | 0.75 |
| E | $FeSO_4.7H_2O$ | -1,0,1 | 0.25 | 0.75 |
| F | Yeast extract | -1,0,1 | 2.5 | 7.5 |
| G | Glucose | -1,0,1 | 50 | 100 |
| H | pH | -1,0,1 | 6 | 7 |
| J | Sodium thioglycolate | -1,0,1 | 0 | 1 |
| K | Cys.HCl: Cysteine hydrochloride | -1,0,1 | 0 | 0.5 |

## Results

*Effect of nutrients in butanol production by Plackett-Burman design*

The results obtained from the P-B design are given in Table 2. According to the F values obtained for the significant variables after performing ANOVA for Table 2, the significant terms are arranged as given in Figure 1. Factors are in order of significance as follows, yeast extract > $MgSO_4.7H_2O$ > glucose> cysteine HCl > pH> $FeSO_4.7H_2O$> $K_2HPO_4$> $CaCO_3$> $(NH_4)_2SO_4$.

The results showed that the variables with probability level greater than 95% were significantly influencing butanol

Table 2: Experimental designs and results of the P-B design for the screening of significant process variables affecting butanol production

| Runs | $(NH_4)_2SO_4$ | $K_2HPO_4$ | $CaCO_3$ | $MgSO_4$ | $FeSO_4$ | YE * | G * | ST* | Cys.HCl* | pH | Butanol |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3 | 1 | 4 | 0.25 | 0.25 | 2.5 | 100 | 1 | 0 | 7 | 5.28 |
| 2 | 1 | 1 | 2 | 0.25 | 0.25 | 2.5 | 50 | 0 | 0 | 6 | 6.51 |
| 3 | 1 | 3 | 4 | 0.25 | 0.75 | 2.5 | 50 | 1 | 0.5 | 6 | 5.82 |
| 4 | 3 | 3 | 2 | 0.75 | 0.25 | 2.5 | 50 | 1 | 0.5 | 7 | 8.19 |
| 5 | 1 | 1 | 2 | 0.75 | 0.75 | 7.5 | 50 | 1 | 0 | 7 | 8.38 |
| 6 | 1 | 3 | 2 | 0.25 | 0.25 | 7.5 | 100 | 0 | 0.5 | 7 | 8.71 |
| 7 | 1 | 1 | 4 | 0.75 | 0.75 | 2.5 | 100 | 0 | 0.5 | 7 | 9.50 |
| 8 | 3 | 3 | 2 | 0.75 | 0.75 | 2.5 | 100 | 0 | 0 | 6 | 6.95 |
| 9 | 3 | 3 | 4 | 0.25 | 0.75 | 7.5 | 50 | 0 | 0 | 7 | 8.05 |
| 10 | 3 | 1 | 4 | 0.75 | 0.25 | 7.5 | 50 | 0 | 0.5 | 6 | 8.75 |
| 11 | 3 | 1 | 2 | 0.25 | 0.75 | 7.5 | 100 | 1 | 0.5 | 6 | 9.02 |
| 12 | 1 | 3 | 4 | 0.75 | 0.25 | 7.5 | 100 | 1 | 0 | 6 | 9.96 |

* YE: Yeast extract, G: Glucose, ST: Sodium thioglycolate, and Cys.HCl: Cysteine hydrochloride. All values are in g $L^{-1}$.

production. The factors yeast extract, $MgSO_4.7H_2O$ and glucose were found significant for butanol production only when all the three were in optimum concentration. Therefore it was necessary to study their interactions which were responsible for increase in butanol production by CCD.

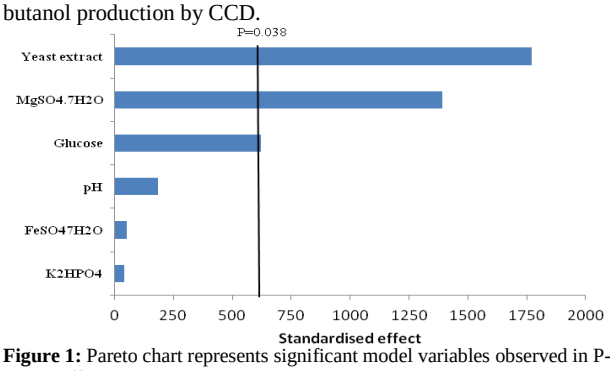



**Figure 1:** Pareto chart represents significant model variables observed in P-B design affecting butanol production

Table 3: Experimental design matrix and results of CCD

| Runs | Glucose | Yeast extract | $MgSO_4.7H_2O$ | Butanol yield |
|---|---|---|---|---|
| | ($g\ L^{-1}$) | | | |
| 1 | 80.00 | 7.00 | 0.50 | 8.22 |
| 2 | 80.00 | 6.00 | 0.70 | 8.03 |
| 3 | 93.41 | 6.50 | 0.60 | 9.32 |
| 4 | 85.00 | 6.50 | 0.60 | 8.83 |
| 5 | 85.00 | 6.50 | 0.60 | 8.71 |
| 6 | 90.00 | 6.00 | 0.50 | 8.38 |
| 7 | 85.00 | 6.50 | 0.60 | 8.54 |
| 8 | 85.00 | 6.50 | 0.60 | 9.13 |
| 9 | 90.00 | 6.00 | 0.70 | 7.84 |
| 10 | 85.00 | 7.34 | 0.60 | 7.79 |
| 11 | 85.00 | 6.50 | 0.60 | 8.96 |
| 12 | 85.00 | 6.50 | 0.43 | 7.35 |
| 13 | 80.00 | 6.00 | 0.50 | 8.31 |
| 14 | 85.00 | 5.66 | 0.60 | 8.06 |
| 15 | 76.59 | 6.50 | 0.60 | 8.14 |
| 16 | 80.00 | 7.00 | 0.70 | 6.96 |
| 17 | 90.00 | 7.00 | 0.50 | 9.06 |
| 18 | 85.00 | 6.50 | 0.60 | 8.69 |
| 19 | 85.00 | 6.50 | 0.77 | 6.09 |
| 20 | 90.00 | 7.00 | 0.70 | 7.22 |

*Interaction of selected factors for butanol production by using CCD*

The experimental design and respective results are given in Table 3. Butanol was in range of 6.08 g $L^{-1}$ to 9.3 g $L^{-1}$. The F-value of 18.69 implies that the model is significant. To explain the butanol production, second-order polynomial equation (1) was established by applying multiple regression analysis on the experimental data.

$$Y = +8.80 - 0.11 + 0.22B - 0.44C - 0.26A^2 + 0.028B^2 - 0.68C^2 + 0.15AB - 0.28AC - 0.11B \quad \ldots\ldots(1)$$

Where Predicted Butanol Production rate is denoted by Y; A, B and C are coded values of yeast extract, glucose and $MgSO_4$ $7H_2O$ respectively.

The results of ANOVA are given in Table 4. Values of "$Prob_{model}$ > F" less than 0.05 (P<0.001) indicate model terms are significant. In this case B, C, $A^2$, $C^2$ and AC are significant model terms while the "Lack of Fit F-value" of 2.17 implies that it is not significant relative to the pure error.

Table 4: ANOVA for response surface quadratic model for butanol production

| Source | Sum of Squares | Mean Square | F Value | Prob > F |
|---|---|---|---|---|
| Model | 11.81 | 1.31 | 18.69 | < 0.0001 |
| A | 0.17 | 0.17 | 2.48 | 0.1463 |
| B | 0.64 | 0.64 | 9.19 | 0.0126 |
| C | 2.66 | 2.66 | 37.94 | 0.0001 |
| $A^2$ | 0.95 | 0.95 | 13.53 | 0.0043 |
| $B^2$ | 0.01 | 0.01 | 0.17 | 0.6922 |
| $C^2$ | 6.70 | 6.70 | 95.44 | < 0.0001 |
| AB | 0.18 | 0.18 | 2.63 | 0.1359 |
| AC | 0.64 | 0.64 | 9.18 | 0.0127 |
| BC | 0.09 | 0.09 | 1.28 | 0.2840 |
| Residual | 0.70 | 0.07 | | |
| Lack of Fit | 0.48 | 0.10 | 2.17 | 0.2079 |
| Pure Error | 0.22 | 0.04 | | |

* $R^2$=0.9439, α (significance level) = 5%.

The determinant coefficient ($R^2$) was 0.9439, indicating 94.39% variability of the response variable. "Adeq. Precision" measures the signal to noise ratio, where a ratio greater than 4 is desirable. The ratio of 16.64 of the model indicates an adequate precision. There is a good agreement between experimental and predicted values which implies that the equation has perfectly described the effect of three factors on the butanol production. The response surface plots are given in figure 2 (A, B and C), which depicts the interactions between two variables by keeping the other variables at zero level.

The quadratic effect of yeast extract and $MgSO_4.7H_2O$ concentrations were highly significant ($P$<0.015), indicating a great impact on the butanol production. However, the quadratic of yeast extract and the interactive effect between $MgSO_4.7H_2O$ and glucose, glucose and yeast extract concentrations were not significant ($P$>0.1), indicating less impact on butanol production.

## Discussion

Microbial fermentation is a process in which the product concentration varies under different conditions. Components of the media play a significant role in metabolism for butanol production. So various factors of the medium were screened by P-B design and then the interaction of the selected factors was studied using CCD.

It is evident from the experimental results of P-B design that $CaCO_3$ and $(NH_4)_2SO_4$ are insignificant with negative coefficients, whereas pH, $FeSO_4.7H_2O$ and $K_2HPO_4$ were found insignificant with positive coefficients. Therefore, these factors were not included in CCD. Cysteine hydrochloride like any other reducing agent is essential for maintaining anaerobiosis, but its level was not increased because of toxic effects of reducing agents at higher concentration (Fukushima et al. 2002).

Yeast extract, a nitrogen source, also provides minerals, proteins, growth factors etc. thereby promoting growth and enhancing butanol production (Al-Shorgani et al. 2013). In the present study increase in yeast extract concentration in the production medium from 5.1 to 7.5 g $L^{-1}$ enhanced the butanol yield from 7.6 to 9.9 g $L^{-1}$. These results were comparable as reported by Canganella et al in 2002 where the increase in yeast extract concentration from 0.05 to 2% enhanced the glucose tolerance but glucose concentrations above 10% caused an increase in doubling time even in the presence of 2% yeast extract

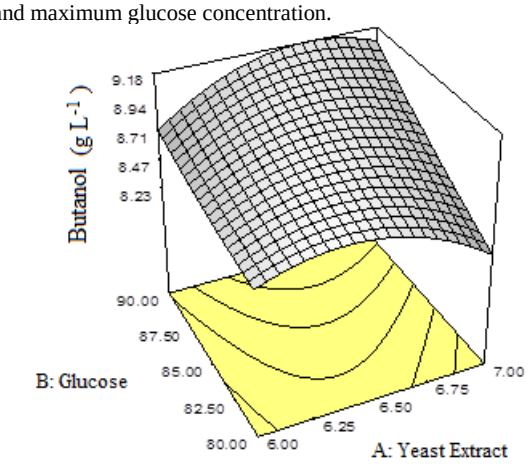
High glucose concentrations have inhibitory effect on butanol production because of inhibition of glucose permease at butanol concentration of 12 g $L^{-1}$ (Ounine 1985). Lee et al. (1993) suggested that initial glucose concentration should not exceed 90 g $L^{-1}$ because it negatively affects the growth. Further Similar results were obtained in the present study where increase in butanol production was observed with the increase in glucose concentration upto 100 g $L^{-1}$. Further He and Chen (2013) showed that at glucose concentration of 100 g $L^{-1}$, highest total solvent production was found, which was 1.39 times of that achieved at 60 g $L^{-1}$ glucose.

The solvent yield (g/g), however decreased with the increase in substrate concentration. As presented in the Table 2, increase in $MgSO_4.7H_2O$ from 0.25 to 0.75 g $L^{-1}$ increased butanol production from 5.28 g $L^{-1}$ to 9.96 g $L^{-1}$. $MgSO_4.7H_2O$ is reported to increase nutrient uptake from media. Pasternak et al. 2010 reported $Mg^{2+}$ to be essential for activation and production of majority of enzymes like ribozymes, endonucleases etc. Mori et al., 1985 showed that $Mg^{2+}$ acts as activator of enzymes like transferases and decarboxylases that play an important role in biochemistry of alcohol production.

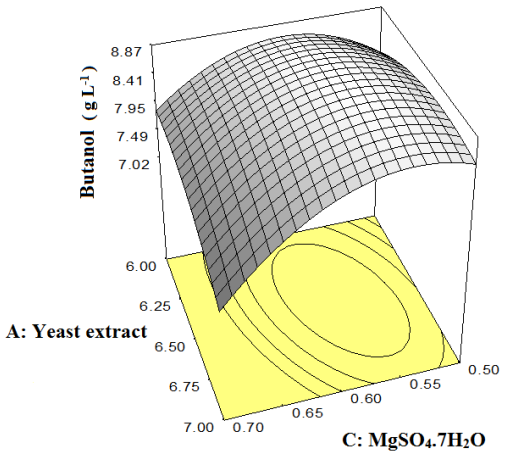
These factors were therefore chosen for CCD to study their interaction with butanol production. After applying this statistical design, equation and response plots were generated. The maximum butanol production of 9.18 g $L^{-1}$ was estimated from equation having yeast extract, $MgSO_4.7H_2O$ and glucose at concentrations of 6.67 g $L^{-1}$, 0.55 g $L^{-1}$ and 90 g $L^{-1}$ respectively.

The butanol production rate increased with increasing yeast extract and $MgSO_4.7H_2O$ concentrations to the optimal levels, and then decreased with a further increase.

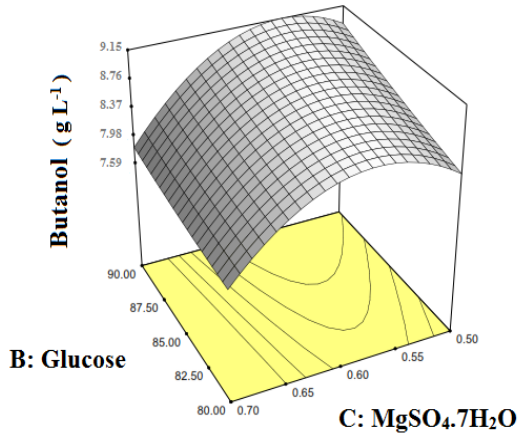
From Fig. 2A, it is clear that the interactions between yeast extract and glucose had maximum effect on butanol production at optimum yeast extract and maximum glucose concentration. In Fig. 2B, response surface plot has a peak which means that the maximum butanol production could be achieved inside the design boundary. In Fig 2C, the effect of $MgSO_4.7H_2O$ - glucose interaction on butanol production was maximum at optimum $MgSO_4.7H_2O$ and maximum glucose concentration.




**(A)**




**(B)**




**(C)**

**Figure 2:** The response surface plot and the corresponding contour plot for butanol production by CHTa as a result of interaction between A) Glucose and yeast extract, with the $MgSO_4.7H_2O$ at level of 0.6 g $L^{-1}$. B) Yeast extract and $MgSO_4.7H_2O$, with the Glucose at level of 85 g $L^{-1}$ C) Glucose and $MgSO_4.7H_2O$, with the Yeast extract at level of 6.5 g $L^{-1}$.

Table 5: Validation of the result obtained after optimization by P-B design.

| Solutions | AS | $K_2HPO_4$ | $CaCO_3$ | $MgSO_4$ | FeSO4 | YE | Glucose | pH | ST | Cys HCl | Butanol (g $L^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | (g $L^{-1}$) | | | | | | Predicted | actual |
| 1 | 1.07 | 1.06 | 3.46 | 0.71 | 0.71 | 6.85 | 85.12 | 6.42 | 0.76 | 0.49 | 10.00 | 10.00 |
| 2 | 2.86 | 1.39 | 3.95 | 0.54 | 0.36 | 7.40 | 99.67 | 6.03 | 0.47 | 0.48 | 9.96 | 10.29 |

* AS: $(NH_4)_2SO_4$, YE: Yeast extract, G: Glucose, ST: Sodium thioglycolate, and Cys.HCl: Cysteine Hydrochloride.

Table 6: Validation of the result obtained after optimization by CCD.

| Solutions | Glucose | Yeast extract | $MgSO_4.7H_2O$ | Butanol (g $L^{-1}$) | |
|---|---|---|---|---|---|
| | | (g $L^{-1}$) | | Predicted | Actual |
| 1 | 90 | 6.79 | 0.56 | 9.16 | 9.61 |
| 2 | 90 | 6.67 | 0.55 | 9.18 | 10.08 |

* Other variables were at zero level for butanol production.

Table 7: Comparison of butanol production by different strains of *Clostridium*

| Microorganism | Initial Glucose concentration (g $L^{-1}$) | Time (h) | Butanol concentration (g $L^{-1}$) | Supplementation | References |
|---|---|---|---|---|---|
| *C.beijerinckii* BA101 | 60 | 60 | 11.90 | PABA, Thiamin Biotin | Ezeji et al. (2003) |
| *C. saccharoperbutylacetonicum* N1-4 | 66 | 36 | 16.20 | No supplementation | Thang et al. (2010) |
| *C. acetobutylicum* CGMCC 5234 | 60 | 85 | 9.58 | P2 Stock solution | Chen et al. (2013) |
| *Clostridium* YM1 (new isolate) | 50 | Not mentioned | 10.93 | Biotin, PABA | Al-Shorgani et al. (2013) |
| *C. acetobutylicum* ATCC 824 | 80 | 72 | 10.10 | Asparagine | Ventura et al. (2013) |
| *Clostridium beijerinckii* strain CHTa | 90 | 48 | 11.80 | No supplementation | Present study |

Thus moderate interactions were observed between yeast extract-glucose, and $MgSO_4.7H_2O$ - glucose. The software suggested few solutions according to the equation for validation of the results. These results for model based solutions that were verified for P-B and CCD experiments are given in Table 5 and 6.

The results clearly indicated good agreement between experimental and calculated values from the model. Although the fermentations were carried out upto 72 h, it was found that butanol amount (11.8 g $L^{-1}$; data not shown) did not increase beyond 48 h. This was mainly due to the butanol tolerance level of the strain CHTa i.e. 1% (w/v) of butanol (data not shown). Like all strains of *Clostridium beijerinckii* strain CHTa also produced other products like acetone (0.16 g $L^{-1}$) and iso-propanol (1.5 g $L^{-1}$).

The medium used in the present study does not have supplements like vitamin, amino acids etc. which were present in media used by Bahl (1986) and Ezeji (2007). Since, these factors add to cost of fermentation, production medium modified for strain CHTa in present study is advantageous. Comparison of different butanol producing *Clostridia* is given in Table 7.

From the Table 7 it is seen that on fermentation time scale, CHTa is second highest strain producing 11.8 g $L^{-1}$ in 48h after *C. saccharoper-butylacetonicum* N1-4 that produces 16.2 g $L^{-1}$ in 36h. *Clostridium beijerinckii* strain CHTa as compared to other *C.beijerinckii* strains produces highest butanol in less time that too without any addition of vitamins or amino acids to the production medium.

## Conclusions

The results of the present study reveal that nutritionally optimized production medium could be used for enhanced butanol production. The optimization of fermentation medium successfully identified glucose, $MgSO_4.7H_2O$ and yeast extract as the key factors responsible for production of butanol.

Using the optimal conditions, the butanol concentration increased to 10.08 g $L^{-1}$ and productivity of 0.45g $L^{-1}$ $h^{-1}$ which was 36.5% and 40% higher as compared to that obtained with un-optimized medium (6.4 g $L^{-1}$, 0.27 g $L^{-1}$ $h^{-1}$) in 24h. It clearly shows the efficacy of optimized medium, giving higher butanol production in less time.

The productivity of butanol was calculated as the butanol in g $L^{-1}$ divided by fermentation time in hour which was expressed as g $L^{-1}$ $h^{-1}$. In addition, strain CHTa can also produce butanol from butyrate, which can be explored for industrial butanol production. The butanol production can further be increased for strain CHTa by either making the culture tolerant to high levels of butanol or by stripping out butanol simultaneously during its production.

## Acknowledgement

Authors would like to acknowledge Department of Biotechnology (DBT), India for financial support. Dr. Amol Mali is gratefully acknowledged for statistical help.

## References

Al-Shorgani NK, Hamid AA, Yusoff WMW, Kalil MS (2013) Pre-optimization of medium for biobutanol production by a new isolate of solvent-producing clostridium BioResources 8:1420-1430.

Bas D, Boyac İH (2007) Modeling and optimization I: Usability of response surface methodology. J Food Eng 78:836-845.

Bahl H, Gottwald M, Kuhn A, Rale V, Andersch W, Gottschalk G (1986) Nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum*. Appl Environ Microbiol 52:169-172.

Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76:965-977.

Canganella F, Kuk SU, Morgan, H, Wiegel J. (2002). *Clostridium thermobutyricum*: growth studies and stimulation of butyrate formation by acetate supplementation. Microbiol res, 157(2), 149-156.

Chen, Y et al (2013) Production of butanol from glucose and xylose with immobilized cells of Clostridium acetobutylicum. Biotechnol Bioprocess Eng 18:234-241.

Cheng CL, Che PY, Chen BY, Lee WJ, Chien LJ, Chang JS (2012) High yield bio-butanol production by solvent-producing bacterial microflora. Bioresour Technol 113:58-64.

Chung KT, Bryant MP, Robert E (1997) Hungate: pioneer of anaerobic microbial ecology. Anaerobe 3:213-217.

Ezeji, TC., Qureshi N, Blaschek HP (2003) Production of acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 and in situ recovery by gas stripping. World J Microb Biot 19:595-603.

Ezeji TC, Qureshi N, Blaschek HP (2007) Bioproduction of butanol from biomass: from genes to bioreactors. Curr Opin Biotech 18: 220-227.

Fouad M, Abou-Zeid AA, Yassein M (1975) The fermentative production of acetone-butanol by *Clostridium acetobutylicum*. Acta Biol Acad Sci H 27:107-117.

Fukushima RS, Weimer PJ, Kunz DA (2002) Photocatalytic interaction of resazurin N-oxide with cysteine optimizes preparation of anaerobic culture media. Anaerobe 8:29-34.

He GQ, Kong Q, Ding LX (2004) Response surface methodology for optimizing the fermentation medium of *Clostridium butyricum*. Lett Appl Microbiol 39:363-368.

He Q, Chen H (2013) Improved efficiency of butanol production by absorbent fermentation with a renewable carrier. Biotechnol for Biofuels 6: 1-9

Lee DM, Eleftherios TP, Petersent DJ, Bennett GN (1993) Metabolic engineering of Clostridium acetobutylicum ATCC 824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic acetone operon. Biotechnol Bioeng 42:1053-1060.

Lin Y, Wang J, Wang X, Sun X (2011) Optimization of butanol production from corn straw hydrolysate by *Clostridium acetobutylicum* using response surface method. Chin Sci Bull 56:1422-1428.

Lopez-Contreras AM, et al (2003) Production by *Clostridium acetobutylicum* ATCC 824 of CelG, a cellulosomal glycoside hydrolase belonging to family 9. Appl Environ Microbiol 69:869-877.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal chem. 31:426-428.

Miller TL, Wolin MJ (1974) A serum bottle modification of Hungate technique for cultiving obligate anaerobes. Appl Microbiol 27:985–7.

Ounine K., Petitdemange H, Raval G, Gay R (1985) Regulation and butanol inhibition of D-xylose and D-glucose uptake in *Clostridium acetobutylicum*. Appl Environ Microbiol 49:874-878.

Pasternak K, Kocot J, Horecka A (2010) Biochemistry of magnesium. J Elementol 15:601-616.

Ranade DR, Gadre RV (1988) Microbiological aspects of anaerobic digestion:laboratory manual. Maharashtra Association for the Cultivation of Science, Research Institute. Pune. India.

Suzuki T, Mori H, Yamané T, Shimizu S (1985) Automatic supplementation of minerals in fed-batch culture to high cell mass concentration. Biotechnol Bioeng 27:192-201.

Tashiro Y, Takeda K, Kobayashi G, Sonomoto K, Ishizaki A, Yoshino S (2004) High butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 in fed-batch culture with pH-Stat continuous butyric acid and glucose feeding method. J Biosci Bioeng 98:263-268.

Tashiro Y, Sonomoto, K (2010) Advances in butanol production by clostridia. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Spain: Formatex. 1383-1394.

Tashiro Y, Yoshida T, Noguchi T, Sonomoto K (2013) Recent advances and future prospects for increased butanol production by acetone-butanol-ethanol fermentation. Eng Life Sci 13:432-445.

Thang VH, Kanda K, Kobayashi G (2010) Production of acetone–butanol–ethanol (ABE) in direct fermentation of cassava by Clostridium saccharoperbutylacetonicum N1-4. Appl Biochem Biotechnol 161:15-170.

Ventura JRS, Hu H, Jahng D (2013) Enhanced butanol production in Clostridium acetobutylicum ATCC 824 by double overexpression of 6-phosphofructokinase and pyruvate kinase genes. Appl Microbiol Biotechnol 97:7505-7516.