Statistical and Media Engineering Approaches to Enhance the Butanol Production From Isolated Microbial Strains

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ARTICLE INFO

Received : 20 June 2019
Revised : 28 August 2019
Accepted : 04 October 2019

Keywords:

Butanol; Fermentation;
Biofuels; Media Engineering;
Clostridium sporogens

ABSTRACT

Anaerobic butanol producing bacteria were isolated, among them *Clostridium sporogens* found to be a potent producer of butanol with the production of 0.1 g/L of butanol. Individual fermentation parameters including inoculum age (10 - 24 hrs), size (1 % - 9 % v/v) were optimized and maximum production was observed on 12 hrs old 8 % (v/v) inoculum. From the single parameter optimization results, for further improvement in the production of butanol, media engineering was done using Plackett-Burman (PB) and Central Composite Design (CCD). Production of butanol increased to 1.5 g/L and the effect of different media components in butanol production is evaluated.

Introduction

Fossil fuel consumption has globally increased over the decades, the alarming rate of environmental impacts associated with the consumption of the non-renewal resources is the major reason that leads towards identification of an alternative source of renewable energy (Garcia et al., 2011). Biofuel have recently emerged as a global competitor in spite of its limitations and could successfully become an energy source (Rusmayer et al., 2019). Among biofuels butanol has a lot of potential including high energy content, and the property of the butanol to effectively blend with the currently available fuels makes it a suitable alternative as energy source for the external combustion engine (Li et al., 2019). Generally Butanol is fermented by Gram positive strictly anaerobic microbe along the ABE Acetone-Butanol-Ethanol fermentation pathway (Vivek et al., 2019). Butanol is a four carbon compound with a molecular formula of C_4H_9OH belongs to the aliphatic group of saturated alcohols. Apart from being an efficient biofuel, butanol is a key intermediate in various chemical reactions having wide application across chemical and textile based industries (Lee et al., 2008). Fermentative production of butanol as gained much attention in recent years and mainly *Clostridium* species belonging to the group of anaerobic fermentation having ABE fermentative pathway was found to be the suitable candidates that are capable of producing

butanol (Qureshi et al., 2001). *Clostridium* is widely considered as a promising candidate for butanol production due to its property to utilize a wide range of sugar monomers from lignocellulosic source and following an anaerobic process can readily decrease the cost associated with the fermentation (Ezeji et al., 2007).

Butanol tolerance among most of the microbes are a limiting factor for biological production and wild type strains are more favorable for butanol production since it has more tolerance (Liu et al., 2017). It was estimated that a final titer value of about 13 g/L could inhibit the butanol production (Jones et al., 1986). The inhibition is mainly due to the toxicity associated with the produced butanol in the media and it disrupts the cell membrane and interrupts the internal pH and decrease the ATP thus reducing the overall uptake of carbon source

The pathway purely follows a stringent anaerobic fermentation strategy and thus our current work was focused on screening of a potential butanol producing anaerobic strain from various environmental samples (Gaida et al., 2016). Five microbial strains producing butanol were isolated and identified. Molecular identification of the samples isolated were carried out and the organism identified belonged to the *Clostridium* species and among the five samples four of the samples were identified to be *Clostridium botulinum* and which was eliminated and the rest of the samples belonging

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to *Clostridium sporogens* were analyzed for its production and was found to produce 0.12 g/L of butanol.

The challenging factor in the present study was to improve the production of the wild type strain. Identifying the key elements that need to be included in the production media that are crucial for improving the butanol production is the main concept of media engineering by statistical approach. Statistical approach was designed using Mini tab 17 software Plackett-Burman (Lin et al., 2011) followed by Central Composite Design. In Plackett-Burman 11 parameters were variably designed for identification of the key media components for the production of butanol. From the results of Plackett-Burman the significant media components were identified and the Central composite analyses of the key components were further optimized and a final production value of 1.5 g/L was obtained. The media optimization provides a clear understanding of various parameters that could play a crucial role in the production of butanol.

2. Materials and methods

2.1. Media and chemicals

All media components and media were purchased from HiMedia, Mumbai, India. Standard of n-Butanol was purchased from Sigma-Aldrich.

2.2. Isolation of butanol producing microorganisms

For the isolation of anaerobic bacteria different samples were collected from various sources (Table 1). Cooked meat medium and reinforced clostridial broth (RCB) was used for the isolation of cultures. 1.0 g of soil sample was added to the culture medium. After 48 hrs of incubation these anaerobic cultures were isolated using anaerobic agar and reinforced clostridial agar (RCA). Isolated cultures and *Clostridium acetobutylicum* NCIM 2856 procured from NCIM, Pune were inoculated into a modified production media containing Glucose- 10.0 g/L, Ammonium acetate - 1.5 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.2 g/L, KH_2PO_4 - 0.5 g/L, K_2HPO_4 - 0.5 g/L, NaCl - 0.01 g/L, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ - 0.01 g/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.01 g/L, Yeast extract - 1.5 g/L, Para amino benzoic acid (PABA) - 0.001 g/L, Biotin - 0.00001 g/L, Thiamin - 0.001 g/L to check the butanol production.

Table 1. Samples collected from various sources.

Sample type	No. of colonies	Positive isolates
Paddy field near NIIST	4	0
Contaminated area near railway track Karakkamandapam	3	0
Thar desert, Rajasthan	2	0
Inside radiator of Ford Figo	2	0
Well soil from Ernakulum	4	0
Soil from Thamarassery	5	0
Termite gut (T1 to T4)	4	4
Contaminant from Chamber (T5)	1	1
<i>Clostridium acetobutylicum</i> NCIM 2856 (T6)	1	1

2.3 Identification of butanol producing cultures

For identification of bacteria, genomic DNA was isolated using genomic DNA purification kit (Origin). 16S rRNA sequence was amplified using universal primer. 27F5'AGAGTTTGATCTGGCTCAG3' 1492R 5'TACGGTACCTGTACGACTT3' AB13600 series sequenced was used in sequence were analyzed with the Gen Bank database through BLAST. Sequencing of the above was done and compared with the nucleotide database.

2.4 Bacterial strain maintenance

The bacterial strains were maintained in 20 % glycerol stocks and regularly sub-cultured in reinforced clostridial broth cooked meat medium.

2.5 Optimization of inoculum age and size

Different age cultures 10, 12, 16, 20 and 24 hrs old cultures and Inoculum size of 2 % to 9 % (v/v) were inoculated to production media and butanol production was estimated.

2.6 Statistical optimization of the production media

a) Plackett Burman design

Plackett burman design is most commonly used method to study the effect of media components for butanol production. Experiment is a two factorial design (+1 and -1) with n variables in n+1 experiment. Concentration of n-butanol served as the only response. Twelve randomized experimental runs were generated by the software Minitab17 to determine the effect of nutrients and pH on butanol production. Fermentation was carried out in 150ml Scott bottles under anaerobic conditions. High and low level of variables used include 1) Glucose (60 g and 10 g) 2) ammonium acetate (2.0 g and 0.5 g) 3) yeast extract (2.0 g and 0.5 g) 4) magnesium sulphate (0.5g and 0.1 g) 5) dihydrogen potassium phosphate (1.0 g and 0.2 g) 6) dipotassium hydrogen phosphate (1.0 g and 0.2 g) 7) sodium chloride (0.1 g and 0.01 g) 8) manganese sulphate (0.1 g and 0.01 g) 9) ferrous sulphate (0.1g and 0.01g) 10) cysteine hydrochloride (1.0 g and 0.2 g) 11) pH (8.0 and 6.0). The effects of each variable were investigated by taking the difference between average of measurements made at higher and lower level.

b) Central Composite Design (CCD)

Minitab software version 17 was used for further optimization of the production media was done. Four parameters such as glucose, Ammonium acetate, MnSO_4 pH were selected for Central Composite Design (CCD). The experimental design contained 31 runs.

2.7 Analytical method

Butanol production was quantified using gas chromatography (Shimadzu GC 2014) equipped with FID detector. The column used for detection was Zebron wax capillary column with nitrogen as carrier gas. Injector temperature and detector temperature was set to 210 °C and 230 °C respectively with column temperature varying from 35 °C to 160 °C (@ 5 °C to 100°C, 60 °C to 160 °C). The retention time for butanol was at 10.8 min.

3. Results and discussion

3.1 Isolation of butanol producing microorganisms

Cultures were grown in RCB and cooked meat medium, and were initially screened for the presence of gas formation. The gas producing cultures were further linked serially. Among the 25 isolated pure cultures, 5 butanol producers obtained. Details were depicted in Table 2.

Table 2. Butanol production by the isolates

Positive isolates	Butanol Production (mg/mL)
T1	0.021
T2	0.020
T3	0.021
T4	0.021
T5	0.127
T6	0.095

Five isolates molecular identification was done and four of the isolates (T1 to T4) were identified as *C. botulinum*, and one was *Clostridium sporogens*. Among the six positive cultures the best butanol production showed by *Clostridium sporogens* (T5) was selected for further studies.

3.2 Optimization of inoculum age and size

Maximum yield of 0.259 g/L butanol was obtained with 12 hrs old culture. When increasing the age of the culture, there is no significant increase in the production of butanol. Inoculum age optimization studies previously conducted also showed age of inoculum is an important factor in production of butanol. Production of butanol from glycerol using *Clostridium pasteurianum* DSM 525 increased after the optimization of inoculum age to 15 hrs (Sarchami et al., 2016). Growth curve analysis of *C. sporogens* showed it reaches its stationary phase at 15 hrs. Production of butanol was observed to be maximum at age of 12 hrs, at its exponential phase (12 hrs). This also explains why maximum production observes at 12 hrs. (Fig. 1)

Fig.1. Impact of inoculum age on production of butanol

12 hrs old culture was used for optimization of inoculum size. When the size of the inoculum increases a gradual increase in the production of butanol was also observed up to 8 % (0.824 g/L) (Fig. 2). When the number of cells increases utilization of glucose also increases and hence the production rate reaches its maximum. Inoculum size has significant importance in the production of butanol was reported earlier by Razak et al., 2011 optimized the inoculum size to 16.20 % for the maximum production of butanol from oil palm decanter cake (OPDC) hydrolysate.

Fig.2. Effect of different inoculum size on production of Butanol

3.3. Statistical optimization of media

A) Plackett-Burman design

Effect of media components on the production of butanol was analyzed here. Ten media components and effect of pH was studied using Plackett - Burman design. Among the studied variables MgSO_4 , FeSO_4 , Cys-HCl, K_2HPO_4 , Glucose, Yeast Extract and pH showed a positive effect on the production of butanol and Ammonium Acetate, MnSO_4 , NaCl, KH_2PO_4 showed a negative effect on the production of butanol. From these results, the significant media components on the production of butanol were identified and according to that the CCD was designed. (Fig. 3)

Fig.3. Effect of various media components on the production of butanol

B) Central Composite Design (CCD)

The optimal level of glucose, MnSO_4 , ammonium acetate, pH and the effects of their interactions on butanol production were further studied by the Central Composite Design (CCD). The complete design matrix and butanol yield are listed in Table 4. Yield of butanol is in the range from 0.027 mg /mL to 1.525 mg/mL. Fig. 4a shows the positive interaction between glucose and pH in the butanol production. Low levels and high levels of pH do not influence the yield of butanol. pH in the range of 8.5 to 11.0 gives the maximum production. In case of glucose the range between 50 g/L to 80 g/L gave maximum butanol production. Further increase in glucose does not have any influence on the yield of butanol. From here identified the maximum substrate utilization of the organism in the production media. Increasing the substrate concentration beyond that level is non economical. Fig. 4 b shows there is no direct interaction between MnSO_4 and pH. MnSO_4 in higher concentration is not showing any influence on the production of butanol. Here also the pH range 8.0 to 12.0 gives the maximum yield of butanol. This is because the butanol production pathway first produces acids and then the solvents. In acidic medium the pH will further decrease after the acidogenic phase, this may be the reason for the higher yield of butanol in basic pH range. Butanol production of wild type strain shows more production than the metabolically engineered organisms (Chen and Liao, 2016). Atsumi et al. expressed the butanol pathway in *E. coli* and got a titer of 0.552 g/L they over expressed 6 genes and 5 genes deleted. *C. cellulovorans* studies by Yang et. al got a production of butanol 1.42 g/L after the over expression of *adhE2* gene. *P. putida*, *B. subtilis* (Nielsen et al. 2009), *L. brevis* (Berezina et al. 2010), *S. elongatus* PCC7942 (Lan et al 2011) all these strains studied for butanol production by metabolic engineering. Comparing the butanol production by these engineered strains and these results; the present study showed a value of butanol. Shen et al got the maximum production of 30 g/L butanol in *E. coli* after metabolic engineering by over expressing six genes and four genes were deleted. Without strain improvement *C. sporogens* producing 1.525 g/L. Wild type strain after media engineering and culture condition optimization obtaining higher butanol titer shows the significance of this study.

Fig. 4 a-b. Contour plot (a) showing the effects of glucose and pH on butanol production by *Clostridium sporogens* plot (b) showing the effects of MnSO_4 and pH on butanol production by *Clostridium sporogens*

Table 3 Plackett Burman design with butanol yield

Run Order	Glucose (g/L)	Ammonium Acetate (g/L)	Yeast Extract (g/L)	MgSO ₄ (g/L)	KH ₂ PO ₄ (g/L)	K ₂ HPO ₄ (g/L)	NaCl (g/L)	MnSO ₄ (g/L)	FeSO ₄ (g/L)	Cys-HCL (g/L)	pH	Butanol (g/L)
1	60	0.5	2	0.1	0.2	0.2	0.1	0.1	0.1	0.2	8	0.837
2	10	2	0.5	0.1	0.2	1	0.1	0.1	0.01	1	8	0.555
3	60	0.5	0.5	0.1	1	1	0.1	0.01	0.1	1	6	0.948
4	60	2	0.5	0.5	0.2	0.2	0.01	0.1	0.1	1	6	0.455
5	60	2	0.5	0.5	1	0.2	0.1	0.01	0.01	0.2	8	0.693
6	60	0.5	2	0.5	0.2	1	0.01	0.01	0.01	1	8	1.236
7	10	0.5	2	0.5	1	0.2	0.1	0.1	0.01	1	6	0.507
8	60	2	2	0.1	1	1	0.01	0.1	0.01	0.2	6	0.471
9	10	0.5	0.5	0.1	0.2	0.2	0.01	0.01	0.01	0.2	6	0.552
10	10	2	2	0.5	0.2	1	0.1	0.01	0.1	0.2	6	0.696
11	10	2	2	0.1	1	0.2	0.01	0.01	0.1	1	8	0.913
12	10	0.5	0.5	0.5	1	1	0.01	0.1	0.1	0.2	8	0.729

Table. 4 CCD with butanol yield

Run order	Glucose (mg/mL)	Ammonium Acetate (mg/mL)	MnSO ₄ (mg/mL)	pH	Butanol (mg/mL)
1	75	-0.2	0.0075	8	1.336
2	60	0.1	0.01	6	0.877
3	75	0.4	0.0075	4	0.043
4	90	0.1	0.01	10	0.698
5	75	0.4	0.0075	8	0.307
6	75	0.4	0.0075	8	0.126
7	75	0.4	0.0075	12	0.046
8	75	0.4	0.0075	8	0.330
9	75	0.4	0.0075	8	0.530
10	60	0.7	0.005	10	1.525
11	90	0.7	0.01	10	0.495
12	105	0.4	0.0075	8	0.358
13	75	0.4	0.0075	8	0.578
14	75	0.4	0.0025	8	0.585
15	75	0.4	0.0125	8	0.601
16	90	0.1	0.005	10	0.315
17	90	0.7	0.005	6	0.431
18	60	0.7	0.01	6	0.227
19	45	0.4	0.0075	8	0.297
20	60	0.7	0.01	10	0.143
21	75	1	0.0075	8	0.543
22	60	0.1	0.005	6	0.089
23	90	0.1	0.005	6	0.330
24	90	0.7	0.01	6	0.227
25	60	0.1	0.005	10	0.166
26	90	0.1	0.01	6	0.027
27	75	0.4	0.0075	8	0.354
28	60	0.7	0.005	6	0.174
29	75	0.4	0.0075	8	0.403
30	60	0.1	0.01	10	0.766
31	90	0.7	0.005	10	0.492

4. Conclusion

Anaerobic butanol producing cultures were isolated and identified. Among the identified cultures *Clostridium sporogens* was used for further studies. Single parameter optimization of inoculum age and inoculum size were done and production was increased to 0.824 g/L. Media engineering by Plackett - Burman and Central Composite Design was done and further improved to 1.525 g/L. Media engineering and culture conditions have a significant effect on the production of butanol. Plackett - Burman and Central Composite Design are reliable and suitable methods in identifying the major factors influencing the production of butanol. Media engineering identified significant factors on the butanol production as glucose and pH. Media engineering resulted in a two fold increase in butanol production. Fine tuning could improve the overall process economics.

5. Acknowledgement

The authors acknowledge the financial support by the Department of Science and Technology (DST), New Delhi under INDO-NORDEN project (Sanction No. DST/IMRCD/INDO-NORDEN/2017(G). Raveendran Sindhu acknowledges Department of Science and Technology for sanctioning a project under DST WOS-B scheme.

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