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Valorization of banana leaves as low cost substrate for the production of butanol

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Abstract

Banana leaves, often disposed off as agro-waste, were used as a low-cost feedstock for butanol production. Steam pretreated banana leaves hydrolyzed by lignocellulolytic enzymes produced by *Sphingobacterium* sp. ksn 11, and obtained 35.36 g/L of fermentable sugars. Phenolic compounds from the banana hydrolysate were removed or detoxified using corn husk residue as an adsorbent. The hydrolyzate was then fermented by *Clostridium acetobutylicum* 2337 (NCIM) and obtained 1.56 g/L butanol, which was further improved upon detoxification and obtained a total ABE of 4.45g/L with 2.28 g/L butanol. Furthermore, addition of 0.55% proline and histidine aminoacids to the fermentation medium increased butanol production to 3.97 g/L and total ABE to 7.57 g/L.

Key words : butanol; banana leaves; proline; histidine; detoxification.

Due to potential physical and chemical properties, butanol now being considered as alternative biofuel which can preferable alternative for gasoline[15]. Industrial production currently being carried out using members of anaerobic solventogenic bacteria such as *Clostridium* genus[10]. Despite the potential advantages for traditional acetone, butanol and ethanol (ABE) fermentation for the production of butanol, there are number of difficulties that has to be resolved, such as cost of substrate and low levels of product concentration etc. Currently, corn starch is being used as substrate which costs around 60 % of overall process[13]. The abundant availability of renewable lignocellulosic biomass is an added advantage, were, barley straw, corn cobs, rice straw, sorghum bagasse, pineapple leaves are now being utilized for the fermentation of ABE[8,13,21]. India being the

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largest producer and consumer of banana, approximately 29 million tons is cultivated annually and around 4 tons are waste being generated consisting of skin, stalks pseudo stems and leaves.Banana waste is used for synthesis biodiesel, biogas, nanocelluloseand other products[1,5,20,27]. During processing and utilization of lignocellulosic biomass, several inhibitors such as acids, phenolic compounds are produced which negatively impact the overall process[12]. Currently, different approaches using resins, charcoal, enzymatic treatment are used, but present need of the hour is effective removal of these inhibitors from medium without losing the sugars[13]. However the conventional production of butanol by solventogeinc bacteria faces additional challenges such as product inhibition low productivity due to the detrimental effect of acids and solvent produced during fermentation[2,3]. To overcome the challenges, several strategies have been incorporated viz., genetic engineering of strain, addition of supplements and cofactors for improvement in microbial growth and production[18]. So far, banana leaves are utilized extensively for ethanol production, less work has been carried out on butanol production utilizing banana leaves as cost effective substrate[19, 28]. The aim of the study is to utilize the banana leaves as low-cost substrate for butanol production and to apply an effective strategy for the removal of phenolic compounds from the fermentation medium and also supplementation of histidine and proline amino acid to the hydrolysate to improve the production of butanol.

Materials :

Glucose, peptone, beef and yeast extracts, ammonium acetate, sodium potassium tartrate, thiamin, sodium carbonate, p-amino-benzoic acid, glycerol, ,dipotassium hydrogen phosphate, L-cysteine hydrochloride, ferrous sulfate, biotin, Folin-Ciocalteu reagent,3,5-dinitro salicylic acid, acetic acid, butyric acid, butanol, acetone, ethanol wereobtained from Himedia chemicals, Mumbai, India.

Banana leaves (BL) pre-treatment and enzymatic saccharification :

The post harvested banana leaves were collected from local agricultural fields, cleaned, dried at room temperature. The dried BL were ground to small size, 1-2 mm and subjected to steam pre-treatment at 121°C for 40 min. The pre-treated BL were washed, dried and used for saccharification. The mixture of crude lignocellulolytic enzymes produced by *Sphingobacterium* sp. ksn 11were used for the saccharification[26]. Post-hydrolysis, hydrolysate was centrifuged for 10 min at 10,000 rpm under 4°C to separate the unhydrolysed solids and saccharified hydrolysate was used for fermentation.The concentration of total sugars (DNS method), protein and phenolics were determined by Lowry's method.

Removal of phenolis (detoxification) from BL hydrolysate :

Phenolics were separated according to the method described by Neelkant *et al*.,[17]. The corn husk residue obtained after the lignocellulosic enzymatic hydrolysis was washed thoroughly until free from sugars. Then the BL hydrolysate is passed through the corn husk residue column and eluted hydrolysate was checked for concentration of total phenolics.

Culture revival and seed culture preparation:

Clostridium acetobutylicum (NCIM 2337) was procured from the National Centre for Industrial Microorganisms in Pune, India. The strain was revived in reinforced clostridial medium (RCM) comprising glucose (5g/L), beef extract (10g/L), peptone (10g/L), sodium chloride (5g/L), yeast extract (3g/L), starch soluble (5g/L), sodium acetate (3g/L), L-cysteine hydrochloride (0.5g/L), and resazurin (0.001g/L). The pH was adjusted to 6.5 using 0.1N HCL/0.1N NaOH. The medium was spurge with nitrogen gas for 10-15 minutes to eliminate dissolved oxygen before being autoclaved at 121 C for 15 minutes. The culture was incubated for 24 h at 37ºC, before being used as a seed culture for fermentation.

Production of butanol utilizing undetoxified and detoxified hydrolysate of banana leaves :

50mL of each hydrolysate containing added salts such as K_2HPO_4 3g/L; KH_2PO_4 2.5g/L;$MgSO_4$0.4g/L;NH_4Cl 1g/L was taken in 250 mL Erlenmeyer flask, inoculated with 5% of *C. acetobutylicum* (NCIM 2337) and incubated at 37ºC for 96 h at 180 rpm. The product was analysed at regular interval of times.

Optimization of proline and histidine supplementation by response surface methodology (RSM) :

The effect of proline and histidine amino acids as supplements on the butanol production was optimized by a statistical tool Response surface methodology (RSM)of Box-Behnken design (BBD). The factors with different variables viz., concentration of proline (0.1 %- 1%) and histidine (0.1 %- 1%) and time of (0-96h) were used (Table-1), the BBD model gave 17 set of experiments which are performed and second order quadratic model was used to determine the most preferable response.

Table-1. Levels of independent variables for Butanol production by fermentation using Banana leaves Hydrolysate

Parameters	Minimum	Maximum
Time	0 h	96 h
Histidine	0.1 %	1 %
Proline	0.1 %	1 %

Analysis :

The fermentable sugars in BL hydrolysate were determined using HPLC (Shimadzu-10 ATVP) with an X-Bridge amide column (Waters, 4.6 150 mm) and a refractive index detector (RID) at room temperature. The mobile phase was acetonitrile:water in a 7:3 ratio with a flow rate of 1 mL min^{-1}.

Acetic and butyric acids concentration was assessed using HPLC(Shimadzu-10 ATVP) with a X-Select HSS T3 column (Waters, 4.6× 150 mm) and UV detector at 210nm. The mobile phase used was acetonitrile: water: o-phosphoric acid (1:9, 0.1%) at a flow rate of 1mL min^{-1} for isocratic elution.

The concentration of solvents acetone, butanol, and ethanol (ABE) was assessed by gas chromatography (Agilent 7890B) outfitted with a flame ionisation detector and DB-ALC column. As carrier gases, zero air (400mL min^{-1})

and a mixture of hydrogen (40mL min^{-1}) and nitrogen (25mL min^{-1}) were used. The temperatures 180°C and 280°C were kept as column inlet and detector temperatures, respectively and initial injector temperature maintained at 40°C and slowly increasing 20°C min^{-1} temperature. For all samples, an injection size of 2µL was used.

Chemical composition and preparation of sugar hydrolysate from pre-treated banana leaves :

Banana leaves being the second largest biomass generated during plant cultivation which can be used for conversion into green fuels viz., ethanol, butanol etc[6,27]. The chemical makeup of BL was presented in our earlier report consisted around 30% cellulose, 33% of hemicellulose and 15% of lignin with other extractives[25]. The high content of cellulose and hemicellulose makes suitable for the production of ABE. To obtain the fermentable sugar hydrolysate, initially BL was steam pre-treated to loosen the complex structure of Lignocellulosic biomasses, further this pre-treated biomass was enzymatically hydrolysed by crude mixture of lignocellulolytic enzymes produced by *Sphingobacterium*sp ksn-11[17,26]. The pre-treated and enzymatically hydrolysed

Table-2. Box-behnken design with independent variables and results

		Factor 1	Factor 2	Factor 3	Response 1
Std	Run	A:Time h	B:Histidine %	C:Proline %	Butanol g/L
6	1	96	0.55	0.1	2.84
15	2	48	0.55	0.55	1.57
2	3	96	0.1	0.55	2.79
10	4	48	1	0.1	1.12
1	5	0	0.1	0.55	0
9	6	48	0.1	0.1	0.87
13	7	48	0.55	0.55	1.57
16	8	48	0.55	0.55	1.57
4	9	96	1	0.55	2.91
8	10	96	0.55	0.55	3.97
17	11	48	0.55	0.55	1.57
14	12	48	0.55	0.55	1.57
12	13	48	1	1	1.58
3	14	0	1	0.55	0
7	15	0	0.55	1	0
5	16	0	0.55	0.1	0
11	17	48	0.1	1	1.16

Table-3.Analysis of Variance (ANOVA) for Regression Model of Butanol Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6.74	9	0.7491	147.68	< 0.0001	significant
A-Time	5.35	1	5.35	1055.69	< 0.0001	
B-Histidine	0.0145	1	0.0145	2.87	0.1343	
C-Proline	0.0345	1	0.0345	6.80	0.0350	
AB	0.0003	1	0.0003	0.0623	0.8101	
AC	0.0109	1	0.0109	2.15	0.1860	
BC	0.0007	1	0.0007	0.1458	0.7139	
A^2	0.2903	1	0.2903	57.23	0.0001	
B^2	0.0667	1	0.0667	13.14	0.0085	
C^2	0.0134	1	0.0134	2.64	0.1480	
Residual	0.0355	7	0.0051			
Lack of Fit	0.0355	3	0.0118			
Pure Error	0.0000	4	0.0000			
Cor Total	6.78	16				

BL hydrolysate consists total sugars of 35.36 g/L of total sugars, among which glucose was around 18.4 g/L, xylose 10.43g/L with 2.82 g/L of polyphenols (Fig. 1). Among the known various pre-treatment methods,the steam pre-treatment method require less energy and environmental friendly, compared with the other traditional methods[8,26]. Additionally, the use of an array of lignocellulolytic enzymes produced by *Sphingobacterium*sp ksn-11 was an added advantageous when compared to using individual cellulases and hemicellulases[26]. The hydrolysate obtained from this pre-treatment and saccharification method was then used for fermentation.

Butanol fermentation using undetoxified and detoxified BL sugar hydrolyzate :

The hydrolyzate containing phenolic compounds obtained from the above step were fermented by *C. acetobutylicum* NCIM 2337 for 96 hours and obtained a total ABE of 2.56 g/L containing 1.56 g/L butanol, 0.89 g/L acetone, and 0.11g/L ethanol (Fig. 2). It was pressumed that the low titer of ABE is due to the presence of inhibitory molecules such as phenolics in the hydrolyzate. As these phenolics inhibit the efficient utilization of sugars by microorganisms[12,29], detoxification is a necessary step in the process. Here, we used corn husk residue obtained after enzymatic hydrolysis as an adsorbent, from which about 57 % (1.6 g/L) of phenolics and 3% (1.06 g/L) of sugars were adsorbed. Different detoxification methods were used such as overliming using calcium hydroxide, sodium hydroxide etc. Further acids were used to lower the pH to neutral and charcoal as an adsorbent. These methods lose more amount of sugars from the medium and the use of chemicals is not economically

feasible[4,7,9]. However, application of corn husk residue as adsorbent in the present study is a green approach and a viable process. The hydrolyzate obtained after detoxification was then fermented and 2.28 g/L butanol, 1.45 g/L octane and 0.72 g/L ethanol were obtained with a total ABE of 4.45g/L along with 3.38g/L acetic acid and 2.88 g/L. of butyric acid. After detoxification, ABE production was doubled. However, still about 4.65 g/L of sugars were not utilized and the acids present in the medium were not converted into solvents (Fig. 3). It is assumed that the presence of acids in the medium have negatively affected the organism causing low yield[14]. Some studies have reported the addition of amino acids to the fermentation medium has a postive effect on the production[11,24].

Effect of supplementation of proline and histidine to detoxidfied hydrolyste :

The concentraiton of proline and histinde amino acids as supplents was optimized by RSM and the maximum and minimum variables of different factors of design are presented in the Table-1. A total of 17 set of experiments obtained form BBD model by RSM showed in table-2. were performed and obtained maximum amount of butanol of 3.97 g/L upon supplementation of 0.55 % of proline and histidine each after 96 h (Supplementary file. Fig. 1.), with total ABE of 7.57 g/L. The quadratic model's statistical validity was confirmed by an ANOVA analysis, which revealed a significant result with a low probability value ($P \leq 0.05$) (Table-3). Furthermore, the analysis revealed that time, histidine and proline concentration levelswas a more significant determinant of butanol production. Along with that acetone and ethanol concentrations were found to 2.51 g/L and 1.09 g/L respectively. Earlier studies have reported that supplimentation of histinde and proline has positive effect on the organism as proline can help to osmotic stress tolerance and maintains the fluidity cell membrane caused by the acids accumulation, where as histidine has effect in the metabolic process[16,30]. The addition of amino acids proved beneficial in increasing butanol production making the organism tolerate to stress conditions.

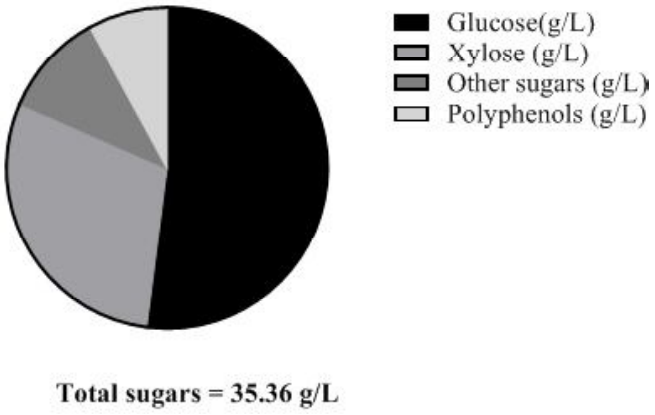


Figure 1. Sugar composition of banana leaves hydrolysate

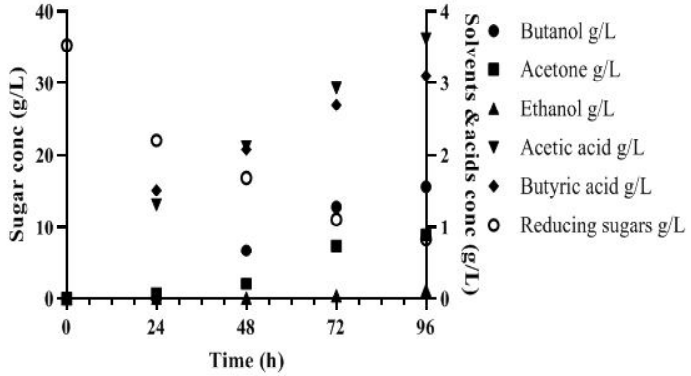


Figure 2. Production profile of ABE from un-detoxified banana leaves hydrolysate

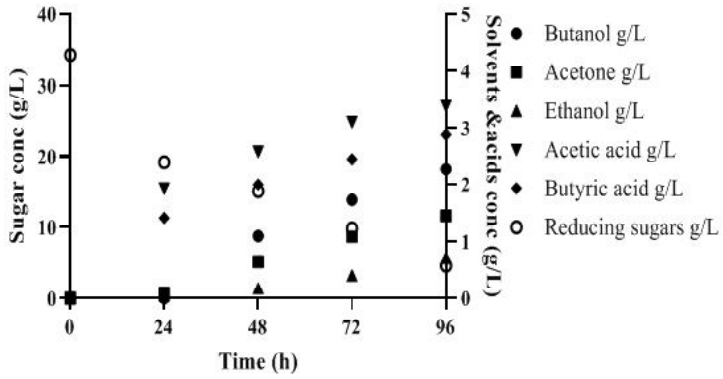
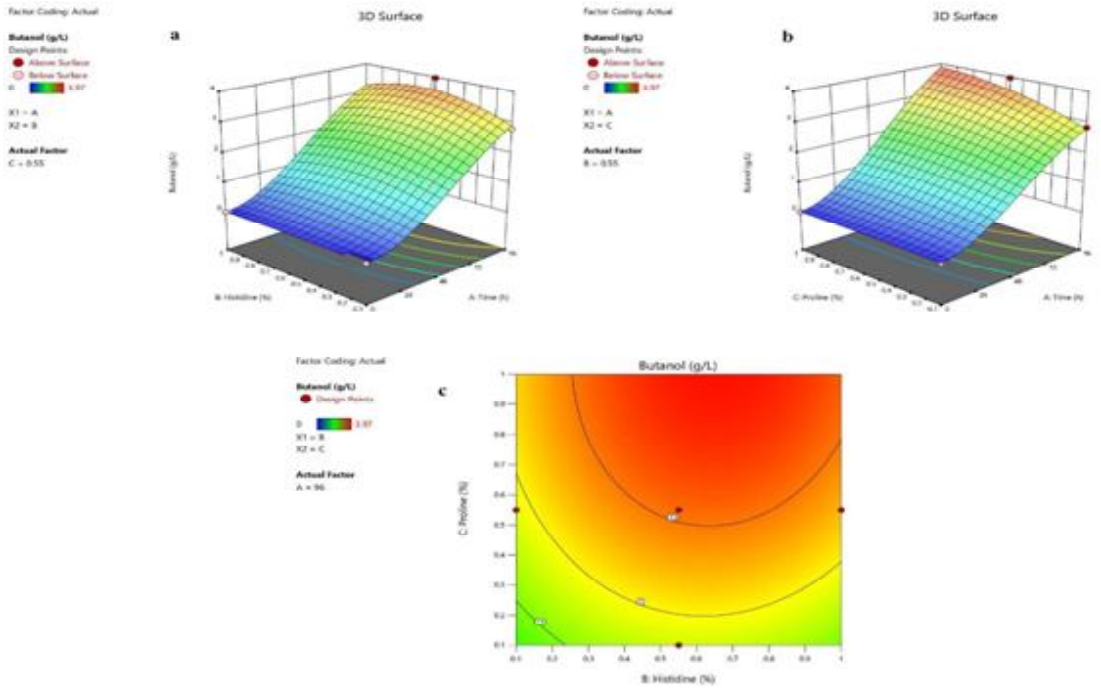


Figure 3. Production profile of ABE from detoxified banana leaves hydrolysate

The results of the present study can be compared with several previous reports, as ABE was produced using different agro-substrates. Sandesh *et al*.,[23] used cocoa pods for production of butanol (3.36 g/L) and 5.27 g/L of total ABE. Salleh *et al*.,[22] reported 2.86 g/L butanol with 5.74 g/L total ABE using oil palm empty fruit bunches. After supplementing the yeast extract to the fermentation medium, they evaluated that 4.13 g/L butanol and 7.33 g/L ABE were produced, which was comparable with the present work. However, they used chemical pre-treatment which is not cost effective and not environmentally friendly. Higher production of butanol (10.11 ± 0.23 g/L) and ABE (15.70 ± 0.31 g/L) was observed when valine and agrinine were added to fermentation medium when commerical glucose was the lone carbon source[18].The production of butanol (4.63g/L) with total ABE of 8.73 g/L was reported from the pineapple leaves[21]. Nevertheless, this study provides an insight into the effective use of banana leaves as a substrate for butanol production, and the phenolic compounds in the hydrolysate are removed from corn husk residue with minimal

Supplementary file

Figure 1. Response surface plots showing the effects on butanol production a. time and histidine, b. time and proline and c. histidine and proline



loss of sugars, makes the whole process was environmentally friendly and cost-effective. Furthermore, the addition of proline and histidine significantly improved stress tolerance and productivity.

This study demonstrated the potential of using banana leaves as a feedstock for green fuel production. Enzymatic hydrolysis of BL resulted in 35.36 g/L of total sugars which was used for fermentation by *C. acetobutylicum* NCIM 2337. However, phenolics present in the hydrolysate were removed by using corn husk residue as an adsorbent, improved the titer from 2.56 g/L to 4.45 g/L of total ABE. Further supplementation of proline and histidne amino acids improved the whole process approximaltely by 50% and produced 7.57 g/L of total ABE. Overall, this study highlights the potential of using banana leaves as sustainable substrate for green fuel production.

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