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Integrated Approach to Biobutanol Production from Sago Spent Waste: Optimisation via RSM and Comprehensive Characterization

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ABSTRACT

Utilizing agricultural waste for biofuel production holds immense significance in the realm of sustainable energy. In particular, biobutanol, as an advanced biofuel, offers a promising alternative to traditional fossil fuels due to its higher energy density and lower environmental impact. The current research presents an integrated approach to biobutanol production from sago spent waste, optimization using RSM, utilising immobilised new Bacillus Strain under anaerobic conditions. The study optimises the process using Response Surface Methodology (RSM) and achieves a significant improvement in biobutanol production reaching 31.96 g/l which is one of highest production yield recorded so far. This yield is 2.4-fold higher than conventional free cells fermentation (12.96 g/l) using similar fermentation conditions. This biobutanol as fuel is estimated to have a cost of approx. $0.8/L which is highly economical compared to conventional petrol $ 1.02/L in India. This study showcases the vast scope for transforming underutilized agricultural residues into valuable biofuels, paving the way for a greener and more sustainable energy future.

1. Introduction

Bio-butanol, a valuable biofuel, has garnered significant attention in recent years due to its potential as a sustainable alternative to conventional fossil fuels (Smith et al., 2020). The production of bio-butanol from renewable resources, such as sago spent waste, represents a promising avenue towards achieving biofuel sustainability (Johnson & Brown, 2019). Renewable energy derived from biomass (Bhatti *et al*., 2008; Kumar *et al.,* 2010) would be cost effective and sustainable alternative methods. Biofuels (solid, liquid and gas fuels) are renewable fuels derived from biological feedstocks and include bioethanol or biobutanol as gasoline equivalent (Kapasi *et al.,* 2010). However, to realize its full potential, it is imperative to optimize the production process and thoroughly characterize the bio-butanol produced. The optimization of bio-butanol production traditionally involves the manipulation of conventional fermentation parameters. Response Surface Methodology (RSM), a statistical tool, has emerged as a powerful technique for achieving this optimization (Wang & Zhang, 2018). RSM allows for the systematic exploration of various parameters to enhance the yield and quality of bio-butanol production. In addition to optimization, a comprehensive characterization of the produced bio-butanol is essential. This characterization provides insights into the fuel's properties, which are critical for assessing its suitability as a renewable transportation fuel (Li et al., 2014). Understanding the chemical and physical properties of bio-butanol is crucial for ensuring its compatibility with existing engines and infrastructure.

This study aims to integrate these critical aspects: the fermentation of bio-butanol from sago spent waste, the optimization of production using conventional parameters with RSM, and the thorough characterization of the resulting bio-butanol. By combining these elements, we seek to advance the sustainable production and utilization of bio-butanol as a viable biofuel option.

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2. Methodology

2.1 Collection and characterization of sago waste

The sago pith waste was collected from local sago processing units located in Salem (11.6643° N, 78.1460° E), Tamil Nadu, India. The sago pith collected was dried, powdered, meshed and stored at room temperature. The physio-chemical characterization of waste like sugar content, pH, electrical conductivity, moisture content, total solids, total volatile solids, ash content, available nitrogen, oxygen content, osmolarity, odour, colour were measured (Ubalua *et al.,*2007).

2.2 Isolation and screening of solvent producers

Sago deep sludge waste samples were collected aseptically from sago waste dump sites of sago processing industries. Similarly, soil samples were also collected from alcohol industries. The collected sludge and soil samples were serially diluted and plated on Thioglycollate agar medium anaerobically in Anaerobic Jar with Anaero-gas pack and incubated at 37°C for 24h-48h. Morphologically different facultative anaerobes were isolated and sub-cultured. Among the various isolates butanol producers were screened by adding Jones reagent (composition in Appendix I) to the plate. The colonies that produce butanol turn dark bluish green were further purified and maintained for further study. The isolates were subjected to further screening process to confirm solventogenic properties. The isolates were inoculated into fermentation media (KH_2PO_4- 0.5 g/L; $MgSO_4.7H_20$- 0.3g/L; $FeSO_4$. $7H_20$- 0.01 g/L; $CaCl_2.6H_20$- 6g/L; Yeast extract- 3.0 g/L; Sodium thiosulphate – 0.5g/L) and incubated for 48h at 37 °C. The supernatant was collected from the fermentation setup and analysed for butanol presence using HPLC (Kumar *et al.,*2014). The mobile phase for HPLC was filtered 0.0025M H_2SO_4 and samples were analysed at a flow rate of 0.6 mL/min in samples at a column temperature of 45°C and sample volume of 20 µL. For the analysis of butanol, the UV detector absorption wavelength was setup at 210 nm. The peak obtained was compared with standard peak. Estimation of butanol using spectrophotometric technique was further performed to screen out butanol producers (Seo *et al.,*2009). The dichromate test enabled to identify the solvent producers and simultaneously estimate the quantity of solvent produced. For the estimation of butanol, a set of known concentration of 5% standard butanol (0.1mL-0.5mL) was taken in test tubes and 1mL of unknown samples in another set of test tubes. The samples were made up to 1 mL and to this 1 mL of 1N potassium dichromate was added followed by 5mL of concentrated H_2SO_4. To the final solution 3mL of deionised water was added and mixed well. The optical density was measured at 610 nm using a UV spectrophotometer. The quantities of butanol by the isolates were estimated using the standard graph.

2.3 Molecular characterization of isolate (Tiquia et al.,2010)

The positive isolates were identified based on their 16S rRNA sequencing technique. The DNA was extracted using an improved phenol chloroform extraction protocol (Sambrook *et al.,*2006) and agarose gel electrophoresis was done to confirm presence of DNA. The PCR product was then sent for 16S rRNA sequencing (Sanger Sequencing method, PAR Life sciences). The nucleotide sequences of 16S rRNA were then compared with the sequences data in the National Centre of Biotechnology information (NCBI) using the basic local alignment search tool (BLAST). The similarity of isolate with NCBI data was obtained and the accession numbers for the sequences were obtained.

2.4 Optimization of butanol production with the conventional parameters using RSM (Mishra S, 2016)

Optimization of butanol production was carried out using various concentration of sago effluent (10% - 50%) in 250 mL serum bottles with 100 mL volume, inoculated with 4 mL inoculum. The bottles were vacuumed to remove oxygen. The effect of non-variable parameter like the pH (3.0 to 9.0 with interval of 0.5), temperature (25ºC to 50ºC with interval; of 3ºC), sago waste concentration (65g - 400g i.e., 20g eqv.-120g eqv. glucose) and electron carrier $CaCO_3$ (0.5g/L- 5.0g/L) were studied with Box Behnken model of RSM using Design Expert 11.0 software. Four parameters that are significant were analysed and the interactions among the factors A, B, C and D were accessed. The factors at three different levels were taken into account and the model replicates were used for the estimation of error and sum of squares. The statistical analysis of the products yielded was performed using Design Expert 11.0. The average production of butanol was taken as dependent variable (response). A variable second order polynomial equation was fitted to the experimental data and resulted in the following model

$$Y= \beta o + \Sigma \beta nXn + \Sigma \beta nnX^2n + \Sigma \beta nmXnXm$$

2.5 Fermentation and Mass production of butanol under optimized conditions

The fermentation was proceeded in 500mL capped bottle with fermentation medium enriched with statistically optimized sago waste concentration, optimized Temperature, optimized pH, optimized electron carrier and optimized fermentation time. This was subjected to scale up process at optimized conditions using fermentation medium developed by optimization. The fermentations carried out in 500 mL Schott bottles with a working volume of 250mL was further taken up to 3L working volume and finally 10L working volume in large fermentation bottles. The setup was fitted with airlock system to enable anaerobiosis in the bottles. The system was vacuumed to remove any oxygen prevailing in the bottles before inoculation of seed culture. The supernatant of respective setup was obtained and potassium chromate estimation was performed to estimate the butanol concentration. The supernatant was analysed using HPLC for confirmation of butanol production. The optimized condition was followed for mass scale up study. Traditional distillation method was employed in the separation process integrated to L-L extraction process. The *in-situ* extraction of butanol using 2-Ethyl hexanol can reduce the inhibition of butanol thereby increasing the yield. The supernatant collected after centrifugation of the fermentation broth was extracted using vigorous shaking for 45 minutes at lab-scale using separating funnel holding

equal volume of both extractant and supernatant. The extractant with butanol was collected for distillation process to obtain pure butanol. The collected pure butanol is stored in airtight bottles for further characterization and study.

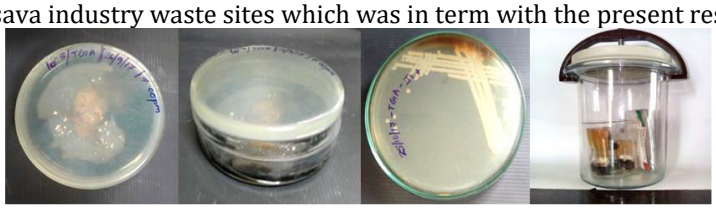
2.6 Characterization of purified product

The purified product is subjected to characterization test to confirm it's purity and identity. The product produced was characterized with gas chromatography – flame ionisation detector (GC-FID), High Performance Liquid chromatography, UV-Vis spectrophotometry, Fourier transform Infrared spectroscopy and Nuclear magnetic resonance spectroscopy.

3. Results and discussion

3.1 Isolation and screening of solvent producers

A total of 40 sago pith waste samples were collected from different sites of four different industries and about 62 different facultative anaerobic isolates were obtained from the waste dump site and incubated at anaerobic conditions (Jar with Anaero gas pack). The isolates were subjected to OF and glucose fermentative test to isolate fermentative strains. Among the sixty-two, 30 isolates were glucose fermenting isolates. Ng and team have reported to have isolated butanol producing bacteria from environmental samples similar to present project (Ng *et al.,*2015). There are corresponding reports of isolation of butanol producing bacteria from the soil (Makut*et al.,* 2018). Johnravindar*et al*., (2017) have isolated butanol producing organisms from cassava industry waste sites which was in term with the present research.



Anaerobic cultivation of isolates in plates | Subculture of positive T1 isolate | Anaerobic jar with Anaero Gas Pack (Himedia)

Fig 1. Isolation of facultative anaerobic isolates

3.2 Analysis of butanol tolerance

The butanol tolerance of the isolates was studied using plate assay and tube assay . The plate assay the isolates C1, T1 and T4 gave a tolerance of 4%, 5% and 2% respectively in Thioglycollate agar media supplemented with various concentration of butanol (1%-10%). The tube assay in Thioglycollate broth media supplemented with various concentration of butanol (1%-10%) gave a tolerance of 3.5%, 5% and 2% with C1, T1 and T4. It was observed that T1 isolate could tolerate about 5% of butanol (Figure 1). The growth OD_{600} of T1 culture decreased from 0.7 ±0.1 (1%) to 0 (6%). It was reported in previous studies that there are several butanol tolerant microbes that can tolerate more than 2% butanol (Bowles *et al.,*1985). Kanno *et al.,* (2013) have shown similar results of butanol tolerance greater than 2%by *Bacillus, Lysinibacillus, Rummeliibacillus, Brevibacillus, Coprothermobacter, Caloribacterium, Enterococcus, Hydrogenoanaerobacterium, and Cellulosimicrobium*. Similar reports were shown by the research done by Kataoka *et al.,* (2011) where *Bacillus subtilis GRSW2-B1* tolerated 5% butanol. The tolerance was observed by a decline in the growth after a particular butanol concentration or the growth inhibition in the butanol medium plates (Vollherbst*et al.,*1984). Sardessai*et al.,*(2002) have also reported 3% of butanol tolerance by *Bacillus sp*isolated from mangroves. There are previous studies showing that the isolated *E. faecium* IB1 showed tolerance to 3% (w/v) isobutanol and *E. faecium*, *E. casseliflavus* showed butanol tolerance up to 2.5% (v/v) butanol (Li *et al.,*2010). There are also reports of *Enterococcus sp* isolated from sago pith waste exhibiting butanol tolerance of 4% (Neethu *et al.,* 2018). Knoshaug*et al.,*(2009) have reported in their study that strains of *E.coli, Zymomonasmobilis*, non-saccharomyces yeasts were unable to tolerate 2% butanol while *Lactobacillus* species could tolerate about 3% butanol (Knoshaug*et al.,*2009). Similar to the present study, Dombek and Ingram (1984) have reported alcohol tolerance of bacteria and attributed the tolerance to the changes in fatty acid profile and fluidity of bacterial cell membrane. Previous reports have stated that the butanol tolerant bacterial membrane composition showed an increase in longer acyl chain fatty acids, indicating an increase in membrane fluidity (Lynd *et al.,*2005).

3.3 Evaluation of solvent production

The isolates were used for fermentation using standard fermentation conditions (T6 medium, 37°C, 48h) and evaluated for butanol production using sago supplemented fermentation medium (Sago supplemented (200g/L) T6 medium, 37°C, 48h). The isolates T1, T4 and C1 were able to produce about 8.16g/L, 7.5g/L and 7.0 g/L of butanol with a productivity of 0.2g/L/h, 0.15 g/L/h and 0.14 g/L/h respectively (Table No 1). Only one isolate (T1) was able to produce higher butanol (8.16g/L) compared to the other two (T4 and C1). The retention time was compared with standard butanol peak and was found to be similar. The sugar concentration in the fermentation medium was also analysed and was found that sugar concentration decreased from initial 60g/L to 5 g/L of sugar. It was found that the isolate was able to produce about 8.16g/L of butanol in sago hydrolysate medium (200g/L - eqv. to glucose: 60g/L) with a productivity of 0.2g/L/h compared to butanol yield in T6 medium at 48h (7.2g/L butanol and productivity of 0.15g/L/h). However, the concentration was lower compared to standard isolate *Clostridium acetobutylicum*(12g/L). Similar reports were also reported with production of 10.38 g /L butanol in a batch process and optimization of the fermentation conditions gave about 12.3 g/L of butanol (Ng *et al*., 2014). Some other reports have stated the production of 10.5 g/L ABE at a ratio of 1:4:11 Acetone:Butanol:Ethanol from cassava waste residue using facultative anaerobes (Johnravindar*et al.,*2019).

Production of butanol from sago hampas was reported previously to be about 4.62g/L (Husin *et al.,*2018). Previous reports by El Hadi *et al.,* (2014) have reported the production of butanol with facultative anaerobic bacteria with about 8.9 g/l in starch medium. The growth curve of the isolate T1 was studied to understand its growth under varying concentration of sugar in the fermentation medium. It can be observed that the isolate tends to utilize some of the sugar for its growth at 24 h fermentation and at 48 h the isolate used up to about 55g/L of sugar in the medium reaching the stationary phase of growth. The growth curve of isolate entered the death phase, the sugar concentration was observed to be around 3.0g/L -2.0g/L (Figure 2). There are previous reports of using HPLC for detection of solvents like ethanol, acetone and butanol similar to the current study (Tsuey*et al.,*2006; Buday *et al.,*1990).

Table 1. Butanol production by the solventogenic isolates

Solventogenic isolates	Butanol concentration (g/L)	
	T6 medium	Sago supplemented T6 medium
T1	7.2	8.16
C1	6.9	7.0
T4	7.0	7.5

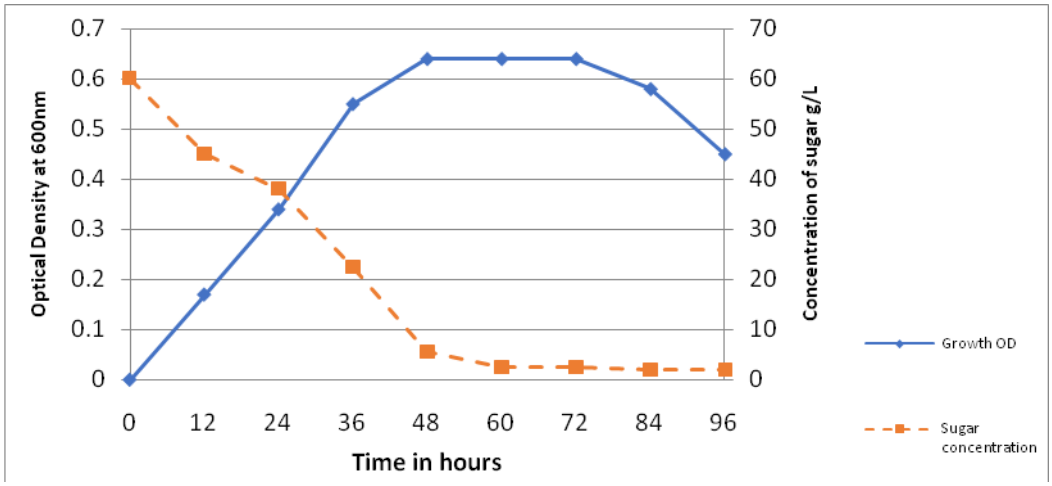


Fig 2 Growth rate of solventogenic bacteria (T1 isolate)

3.4 Molecular characterization of solvent producer

The potent isolate T1 was exposed to morphological and biochemical characterization and finally 16SrRNA sequencing to identify the isolate. The 16SrRNA sequencing results was fed to NCBI Blast and found to be 98% similar to *Bacillus subtilis* and the accession number was obtained MH 814998 (Figure 2).Duan *et al.,*(2013) have reported fermentation of facultative anaerobe *Bacillus sp TSH1* to produce butanol. Romero *et al*., (2007) have reported in their study that wild strain*Bacillus subtilis* does not produce ethanol or acetate. There are previous reports of isolation of butanol producing *Enterococcus sp* from waste (Neethu *et al.,*2018, Ting *et al.,*2012). Serrano *et al.,* (2017) have stated that several Clostridial species like *C. beijerinckii, C.diolis, C. felsineum, C.pasteurianum, C.puniceum, C.roseum, C. saccharoperbutylacetonicum*are solvent producers (Serrano *et al.,*2017). Chantarasiri *et al.,*(2015) have reported isolation of cellulolytic *Bacillus sp*capable of solvent production. *Bacillus sp* capable of producing butanol can be isolated from soil sample (Ng *et al.,* 2014). Shalinimol*et al.,*(2016a) has reported the isolation of *Bacillus sp*from sago industrial waste that has the production of amylase. El-Hadi *et al.,*(2013) have also similarly isolated butanol producing *Bacillus sp*from soil. Wang *et al., (*2015) have reported that *Bacillus cereus* has the ability to enhance the butanol yield.

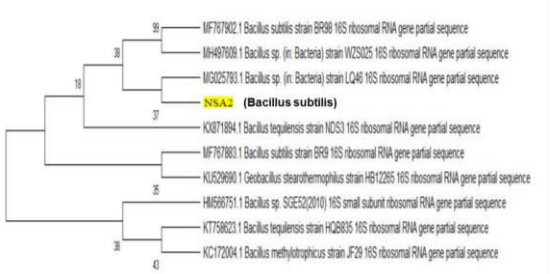


Fig 3 Phylogenetic tree as obtained from NCBI

3.5 Determination of bacterial growth

Cell biomass was estimated after 24 h for treated sago solid waste and 0.74 OD was observed at 600 nm with UV-Vis spectroscopy. Maximum growth was observed after 48-72h in sago pith waste (1.85 OD at 600nm). The bacterial growth in simple Thioglycollate medium was 0.60 OD after 24h. There are previous reports by Ni *et al.,* (2011) discussing on the

effect of parameters like pH, temperature, nitrogen ratio, chemical reactions on the negative culture growth (Ni *et al.,*2011). They also indicated that pH adjustments using NaOH and HCl could deplete cell growth. However, in this study there observed no growth hindrance due to the presence of alkali or acid. Ruban *et al*., (2013) have reported the efficient growth of *Bacillus sp* and *Aspergillus sp* and high amylase production in sago effluent due to its high amount of organic material. Shalinimol, (2016) has reported similar competent growth in sago industrial waste.

3.6 Optimization of butanol production using Response Surface Methodology

In this study, the effect of factors pH, temperature, sago pith waste released sugar and electron acceptor $CaCO_3$ were selected as factors in the Box-Behnken Design (Table 2). The values (low level and high level) for parameters were selected based on the observations obtained from conventional optimization data. As a response butanol concentration was chosen, a total number of 29 experiments were employed for response surface methodology and order of experiments was arranged randomly using the Design Expert 11.0 software.

Table 2. Coded and actual values of variables of the experimental design

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	pH	6.50	5.50	7.50	0.0000	Actual
B	Temperature	34.50	32.00	37.00	0.0000	Actual
C	Sago sugar conc.	70.00	60.00	80.00	0.0000	Actual
D	Electron carrier $CaCO_3$	2.50	1.50	3.50	0.0000	Actual

From the experimental data, according to the design, a second order polynomial regression model equation was derived as below:

$$\mathbf{Y = +12.12 + 0.5675A + 0.4908B + 0.2417C + 0.2383D + 0.8250AB + 0.7250AC + 0.4475AD + 0.0500CD - 4.07A^2 - 4.14B^2 - 3.74C^2 - 4.31D^2}$$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.Two way interactions such as three-factor complexed system are considered for model adequacy. Statistical data were fitted to models such as Linear, interactive, quadratic and cubic to achieve the regression equations. The best suited model for analysing butanol concentration was chosen by lack of fit test and model summary statistics. All $P > 0.05$ for lack of fit signifies the model does not fit the data .Cubic model was neglected whereas quadratic model was found to have maximum "R squared" and "Adjusted R-squared "values. In this study quadratic model was chosen as an adequate model for comparative precision in predicting future consequences and for further analysis. The ANOVA for the response is shown in Table 3. It shows that the quadratic effect of pH (A), Temperature (B), Sago sugar concentration (C), $CaCO_3$ (D) and the model is significant ($p< 0.05$). Regression equation determines the major changes in response of the values and greater F value implies the best suited model.

Table 3. ANOVA for Quadratic model report for Response 1: Butanol concentration

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	289.08	14	20.65	5.82	0.0011	significant
A-pH	3.86	1	3.86	1.09	0.3141	
B-Temperature	2.89	1	2.89	0.8156	0.3818	
C-Sago sugar conc	0.7008	1	0.7008	0.1977	0.6634	
D-Electron carrier CaCO3	0.6816	1	0.6816	0.1923	0.6677	
AB	2.72	1	2.72	0.7680	0.3956	
AC	2.10	1	2.10	0.5931	0.4540	
AD	0.8010	1	0.8010	0.2260	0.6419	
BC	0.0900	1	0.0900	0.0254	0.8757	
BD	0.2550	1	0.2550	0.0719	0.7924	
CD	0.0100	1	0.0100	0.0028	0.9584	
A^2	107.69	1	107.69	30.38	< 0.0001	
B^2	111.02	1	111.02	31.32	< 0.0001	
C^2	90.89	1	90.89	25.64	0.0002	
D^2	120.40	1	120.40	33.96	< 0.0001	
Residual	49.63	14	3.54			
Lack of Fit	20.94	10	2.09	0.2920	0.9480	not significant
Pure Error	28.69	4	7.17			
Cor Total	338.71	28				

Factor coding is **Coded**. Sum of squares is **Type III - Partial**

The **Model F-value** of 5.82 implies the model is significant. There is only a 0.11% chance that an F-value this large could occur due to noise. **P-values** less than 0.05 indicate model terms are significant. In this case A^2, B^2, C^2, D^2 are significant

model terms. Values greater than 0.10 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 0.29 implies the Lack of Fit is not significant relative to the pure error. There is a 94.80% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good- model to fit.

3.7 Interaction effect

To study the effect of parameters pH, temperature, sago sugar concentration and electron carrier CaCO3 on butanol concentration the response surface methodology was adopted, and three-dimensional diagrams were plotted. The three-dimensional diagrams showing the parameter interaction is depicted below (Figure 4).

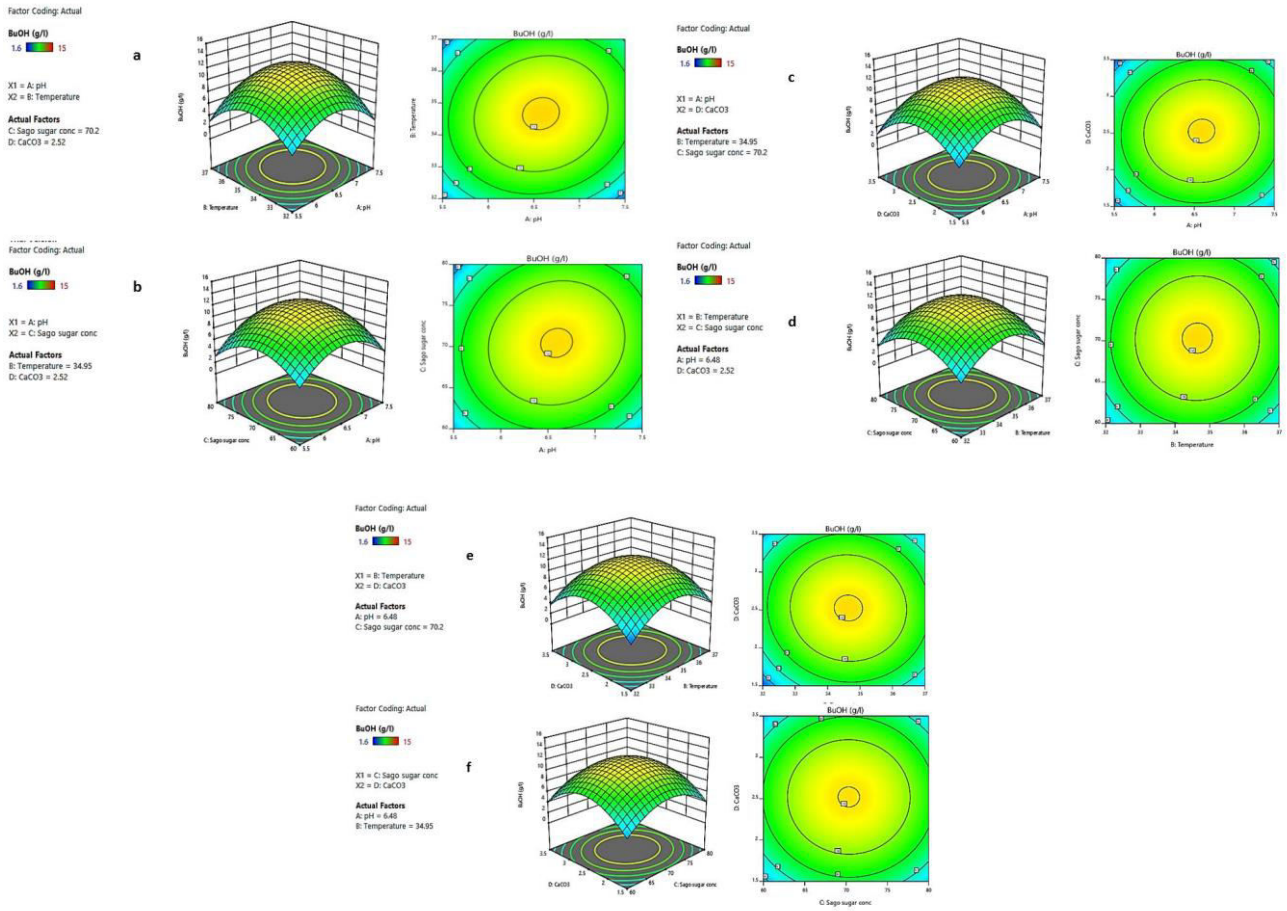


Fig 4 3D response surface graph and contour graph for butanol concentration for a) pH versus temperature b) pH versus Sago sugar conc c) pH versus CaCO3 d) temperature versus sago sugar conc e) CaCO3 versus temperature f) CaCO3 versus sago sugar conc

There observed an increase in butanol concentration from the optimal conditions which was obtained by the method of point prediction and response of plots. The maximum butanol concentration was found to be 12.12 g/L at a pH of 6.5, temperature 35°C, Sago released sugar of 70g and $CaCO_3$ 2.5g/L. Since the experimental values are closer to the predicted ones and p value<0.05 proved that the results confirms the model validity.

Table 4. Optimum and confirmative values of process parameters for maximum butanol concentration

pH	Temperature °C	Sago sugar conc g/L	CaCO3 g/L	Butanol conc. g/L
6.5	34.5	70	2.5	12.12

There are previous reports of employing RSM model for response –butanol production versus several factors glucose concentration, butyric acid and C/N ratio. The model validation experiment showed 12.12 g/L butanol production under optimal conditions in their study (Al-Shorgani*et al.,*2018). Previous studies have also stated using RSM model to study the effect of factors butyric acid, yeast extract and ammonium acetate to give a butanol concentration of 11.06 g/L and a concentration of 11.77g/Lbutanol when performed under optimal conditions obtained from RSM model (Khunchantuek*et al.,*2017).

3.8 Confirmation optimisation study using RSM derived optimal factors

The biobutanol fermentation was performed using RSM derived optimal factors namely pH 6.5, temperature 35°C, 2.5g/L $CaCO_3$ and using 70g sago sugar substrate. The fermentation product was extracted, purified and analysed for butanol

concentration using HPLC and a maximum of 12.96 g/L butanol was produced with a production yield of 0.18g/L/h. The production concentration almost synchronized the RSM confirmation run. The optimal factors were used for further fermentation studies.

3.9 Fermentation of biobutanol

Potent isolate both free cells and immobilized cells using calcium alginate technique at a concentration ratio of 4% sodium alginate and 2.5% $CaCl_2$ for fermentation**.** It was observed that with immobilized cells the concentration of butanol was higher in fermentation medium. The bacterial entrapped cells were able to produce a butanol concentration of 15.03**±**0.04g/L at fermentation under optimized conditions. The cells could be re-used for two to three times. The results were better in comparison to butanol obtained from optimization of medium (12.96±0.1 g/L) and traditional biobutanol production method using free cells(8.16±0.06 g/L).Similar results were reported previously which produced about 11.1 g/L of butanol from xylose with immobilized cells compared to 8.48 g/L butanol which was 28.3% higher to suspended cells (Chen *et al.,*2013). Krasňan*et al.,*(2018) have also reported that entrapped cells could increase the butanol concentration by 6.3 times than free cells using pure glycerol due to the protection of microorganism by gel matrices from toxicity of product.Börner*et al.,*(2014)have reported that unlike the free cells, the immobilized cells gave 2.7 fold increase of butanol concentration 18.2 g L^{-1}. It was observed that implementation of immobilised cells for fermentation caused the butanol concentration to increase to about 15.03g/L from 8.16 g/L with free cells. The immobilized cells were also reported by them to be used from 3 to 5 times. Häggström *et al.,* (1981) has reported the implementation of immobilized spores of *Clostridium acetobutylicum* in a calcium alginate gel.to obtain 67 g Butanol/L-day and achieve continuous Butanol production for 1000 h. Frick *et al*., (1986) has also stated similar results of enhancing butanol concentration using calcium alginate immobilized cells where the ABE production increased from 1.93 g/L/h to 4.02 g/L/h productivity. The recycle of immobilized spores were reported to afford an average ABE concentration of 19.31 g/L from the third run (Shafei *et al.,* 2002).

Table 5 Bio butanol production at different fermentation conditions

	Optimised medium		**Standard medium**	
	Immobilized cell	**Free cell**	**Immobilized cell**	**Free cell**
Butanol concentration	15.03±0.04	12.96±0.1	10.06±1.4	8.16±0.06
Reduced sugar	0.0258±1.2	0.0380±0.85	0.0330±0.15	0.0434±1.2

3.10 *Characterization of the purified butanol*

3.10.1 *FTIR analysis*

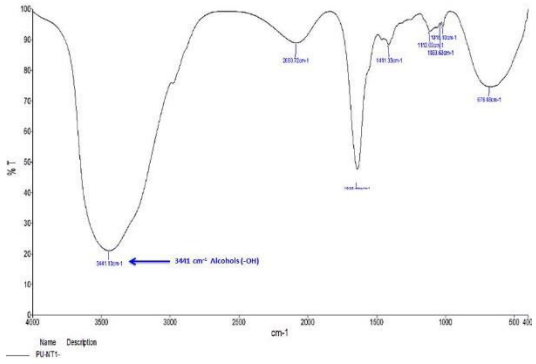


Fig 5 FTIR graph of butanol sample

The FTIR results of the product showed broad peak at 3441 cm^{-1} which denotes the presence of OH-functional group of alcohols (Figure 5). This resembled the standard butanol FTIR graph giving a broad peak at range of 3200-3640 cm^{-1}.The standard chart for FTIR functional group is provided in Appendix I. Similar reports have been stated by Kansiz*et al.,*(2001) where FTIR was used to detect acetone, butanol and ethanol in the fermentationmedium. Schuster *et al.,*(1999) have also used FTIR to detect butanol and similar results were obtained. Xu *et al.,*(2016) have also reported the peak range of 3000-3600 cm^{-1} detecting OH group presence and absorbance at 1315 cm^{-1} and 1730 cm^{-1} indicating the CH and CO groups respectively. Yadav *et al.,*(2014) have reported the formation of peaks at 2858,2927 and 2958 cm^{-1} indicating the hydroxyl groups. Doroshenko *et al.,* (2013) have also reported FTIR spectrum of liquid butanol. Kizil *et al.,* (2002) have also reported similar reports stating the FTIR peaks of O-H (3000-3600 cm^{-1}) stretch, C-H (2800-3000 cm^{-1}) stretch, the skeletal mode vibration of the glycosidic linkage (900-950 cm^{-1}).

3.10.2 NMR analysis

The NMR analysis of sample gave a clear picture of structural changes of molecule. The number of signals gives the different set of protons in the molecule, the position of signal gives the magnetic environment of each set of protons and the area of peak gives the number of protons in each set. The chemical shift can be determined from NMR spectrum and it gives information on the structural environment of nuclei that produced the signal. Alcohols have a standard chemical shift ranging from 0.5 ppm – 6.0 ppm. The spectrum of sample produced five signals indicating four distinct protons respectively with chemical shift at approximately 0.95 ppm, 1.4 ppm, 1.55 ppm, 1.7 ppm and 3.7 ppm. Determining the relative area of the signals it was observed that signal at 3.7 was formed from OH group as it contains electronegative O atom. The signal at 1.7 ppm is formed due to the $-CH_2$ methylene group which is the C2 of the molecule. At 1.55 ppm the signal produced is of C3 methylene group with multiplet peaks. The C4 group forms a peak at 1.4 ppm and the C5 is a triplet signal formed at 0.9 ppm (Figure 6). Joining the fragments together it produced butanol molecule as illustrated in Figure 5.47. Similar results were reported by Yadav *et al.,*(2014) using NMR analysis to confirm the purity of butanol.

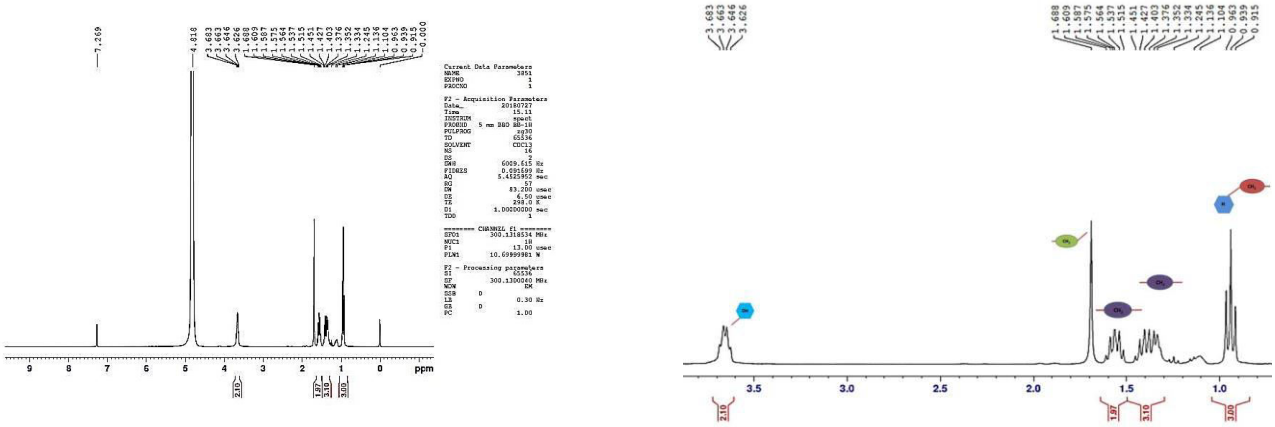


Fig. 6 NMR analysis

3.10.3 GC-FID

The sample analysed with GC-FID (Agilent 6890N) gave sharp peak at retention time 5.011 and a minute peak at retention time 13.041. The sample was compared with standard butanol (Sigma, HPLC grade) and the retention time observed was similar to sample (RT 5.253) and minute peak was identified as 2-Ethyl hexanol (Figure 7). Potter *et al.,*(1996) have also reported similar results of GC-FID analysis for butanol. The present results are supported by the results reported by Lin *et al.,*(2013) to validate the ABE fermentation products.

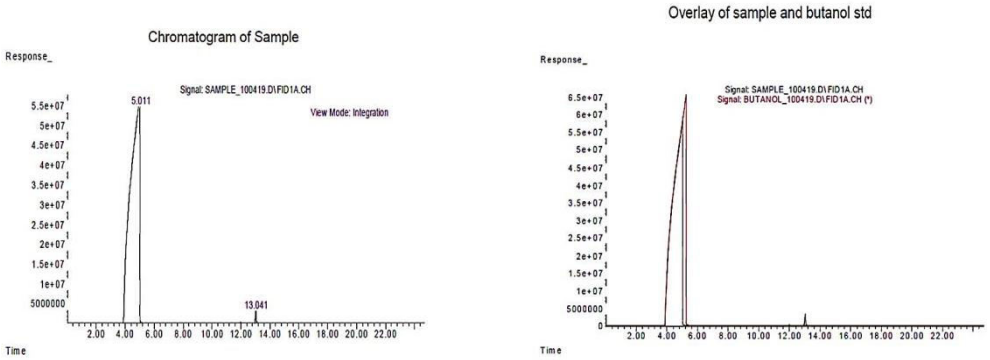


Fig 7 GCFID chromatogram showing peak of purified butanol (Left) and overlay of standard and sample (Right**)**

4. Economic analysis

The biofuel thus produced from sago waste is estimated to have a cost of approx. Rs. 54/L (approx. $0.8/L) which is highly economical compared to conventional petrol Rs.73.36/L ($ 1.02/L) as per December 2018 (using Ms Excel 2010). Joseph et al., (2023) recently have reported a total capital invested per gallon of bioethanol at around USD 17 in sugar cane bagasse and USD 12 in brown algae processes fuel. .

5. Conclusion

This project has yielded promising and positive results. These finding underscore the importance of utilizing waste materials and also highlight the significance of optimisation strategies in enhancing biofuel production processes. This endeavour signifies a significant step forward in the realm of renewable energy, emphasizing the importance of innovative and eco-friendly solutions for a greener future. Implementation of facultative anaerobe represents a notable adaptation. This has enhanced the feasibility of large scale biobutanol production making the approach more practicable and efficient.

Also incorporating immobilization of cells in the biobutanol production signifies a strategic approach. This method not only optimises the efficiency of the production but also allows for better control over cell activities and improved biobutanol production. By utilising such methods the project ensured a more robust platform for sustainable biobutanol production aligning the broader goals of environmental conservation and renewable energy development. The research can be further taken to strain improvement to enhance butanol production for further commercial applications.

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6.1 Conflict of interest

The author declare that there is no conflict of interest.

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