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Production of n-Butanol from Biomass by Two Step Fermentation Process Using *Staphylococcus Sciuri*

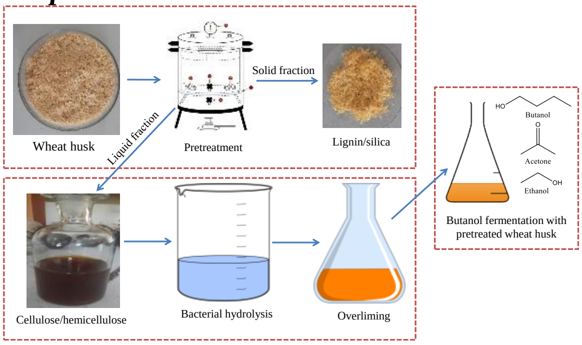
Mukta Rajotia[1], Sudesh Kumar[2], Bechan Sharma[3,*]

Abstract

This study explores the sustainable production of n-butanol from wheat husk through a two-step bioconversion process. Wheat husk, an abundant agricultural residue, was first fermented into butyric acid using Bacillus subtilis. The resultant butyric acid was then converted into n-butanol using Staphylococcus sciuri. The process leverages biological fermentation to create value-added chemicals from lignocellulosic biomass, offering an eco-friendly alternative to petrochemical methods. Two steps fermentation process were carried to increase the final yield of the product as well as to check the effect on n-butanol yield with butyric acid. Gas Chromatography-Mass Spectrometry (GC-MS) was used to verify both the production and purity of n-butanol. The findings suggest that wheat husk can serve as an efficient feedstock for n-butanol production, potentially contributing to biofuel and green chemical industries. Also, the results show that with optimizing the fermentation parameters, we can obtain maximum yield of butanol from butyric acid fermented from wheat husk.

Keywords: n-Butanol, wheat husk, *Bacillus subtilis*, lignocelluloses biomass, *Staphylococcus sciuri*

Graphical Abstract



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INTRODUCTION

Butanol has emerged as a crucial platform chemical for producing a diverse range of industrial chemicals and is increasingly viewed as a superior biofuel alternative to gasoline. Its higher energy density, hydrophobic properties, and enhanced blending capabilities with gasoline make it particularly attractive [1, 2]. Efforts in research aimed at the efficient production of butanol from sustainable and renewable carbon sources have been steadily advancing. Utilizing advancements in biotechnology and process engineering, novel fermentation techniques are being pioneered to transform readily available biomass into butanol

[3]. Historically, butanol has been manufactured using acetone-butanol-ethanol (ABE) fermentation processes involving molasses or starch-based materials and solvent-producing *Clostridium* species [4]. *Clostridia* species are uniquely capable of extracellularly producing biobutanol during the solventogenic phase of ABE fermentation. Various strains, such as *C. saccharobutylicum*, *C. acetobutylicum*, *C. beijerinckii*, and *C. saccharoperbutylacetonicum*, have been extensively researched for their biobutanol production capabilities [5–8].

Production of butanol by *Clostridium acetobutylicum* through acetone-butanol-ethanol (ABE) fermentation yields acetone, butanol, and ethanol in a ratio of 3:6:1. However, there are significant challenges with ABE fermentation, with *Clostridia* species including the anaerobic nature, which complicates their handling and the low yield of butanol due to its high toxicity to the cells [9]. To address these issues, various heterologous hosts, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Pseudomonas putida*, *Bacillus subtilis*, and *Lactobacillus brevis* have been engineered for butanol production. However, none of these engineered hosts could tolerate butanol concentrations beyond 2.0% (v/v) while maintaining considerable growth. The steps involved in bacterial fermentation of ethanol and butanol from WH are shown in Figure 1.

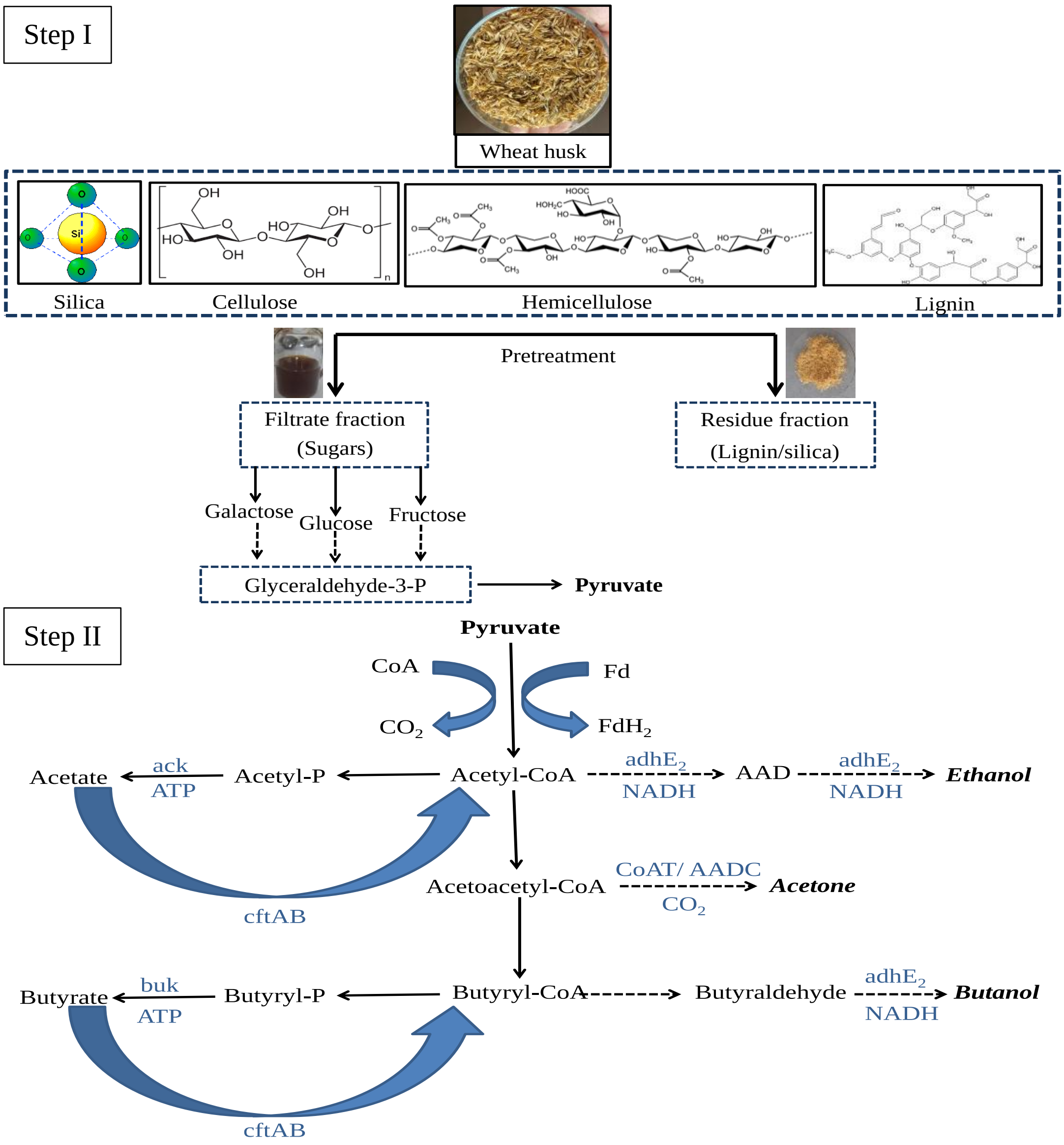

Figure 1. Steps involved in bacterial fermentation of butanol from WH.

Subsequently, natural microbes, such as *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas* species were isolated, which could tolerate butanol concentrations of up to 2.25%, 3.0%, and 6.0% (v/v) respectively [10–12]. Despite this, their growth drops significantly beyond 2.0% (v/v) butanol, indicating the persistence of the problem. In 2019, Liza Goyal et al. discovered a novel *Staphylococcus sciuri* strain KM16, isolated from soil, which could tolerate butanol up to 2.25% (v/v) while achieving relatively higher growth compared to other reported microbes [13].

Same as other bioprocesses, the production of butanol via fermentation is constrained by high substrate costs, low product yields, and expensive recovery processes [14]. To date, numerous renewable materials, including wheat straw, corn stover, and corn fiber, have been utilized for butanol production [15]. However, since no existing microorganism can effectively break down lignocellulosic biomass to produce butanol on its own, a combination of physical-chemical pretreatment and enzymatic hydrolysis is necessary [16]. During the pretreatment process, the biomass undergoes harsh conditions, including high temperatures, and is treated with a range of chemicals. However, some inhibitors are produced during pretreatment process which affects the fermentation process resulting in low yield of final product [17]. Removal of these inhibitors from hydrolysate can increase the production cost. ABE production by *Clostridia* is a biphasic process comprising of acidogenesis and solventogenesis. In acidogenesis, sugars are metabolized to produce acetic acid and n-butyric acid, resulting in lowering of pH to acidic condition. During solventogenesis, the acids are reutilized to produce n-butanol, acetone and ethanol with increase in pH. One of the feasible strategies to reduce toxicity and improve the yield is to first ferment biomass into n-butyric acid. The acid is then converted in the second stage to n-butanol. The concentration of n-butyric acid is very important for the shift from acidogenesis to solventogenesis [18]. David Ramey (1998) was the first to report two stage ABE fermentation reactions. He carried out n-butanol production by first producing n-butyric acid and then inducing metabolic shift to n-butanol [19].

For sustainable industrial-scale butanol production, lignocellulosic biomasses are promising feedstock. Conversion of lignocellulosic materials to fermentable sugars requires pretreatment to affect the structure of lignocellulose by degrading hemicellulosic polysaccharides and increasing the enzyme’s accessibility to cellulose. Cellulose and hemicellulose part in lignocellulosic biomass are protected by lignin complex structure. The rigid structure of lignin makes it difficult for enzymatic hydrolysis to excess and break down the cellulose and hemicellulose into fermentable sugars [20]. Without removing or lowering lignin content significantly reduces the efficiency of enzymatic hydrolysis resulting in low sugar yields [21]. The effect of lignin presence on butanol production by *C. acetobutylicum* has been investigated with cellobiose as the carbon source, in which they found lignin (1 g/L) delayed and decreased butanol production and promoted the accumulation of acetic and butyric acids [22]. Diluted acid pretreatment is highly efficient in the pretreatment of lignocellulosic biomass. It has also been applied for the hydrolysis of starch to glucose. According to previous studies, a diluted acid pretreatment is a promising process for biomass containing both lignocellulose and starch [23].

Other than the pretreatment process, the choice of fermentation substrate also plays a crucial role in determining the cost of butanol production. Therefore, it is essential to identify substrates that are readily available, rich in fermentable sugars, structural carbohydrates, which can be hydrolyzed using inexpensive and straightforward pretreatment methods to make them a good candidate for butanol fermentation [24]. Wheat husk (WH), a renewable by-product from flour industries, is produced in substantial quantities, with around 761 Mmt (million metric tons) generated annually (World agricultural production, US Department of Agriculture (USDA, pp. 19, 2019). The total wheat production in India in 2017/2018 was 97.11 Mt [25]. The total surplus crop residue produced in India is 82 Mt (metric tons) in which 70 Mt is burned annually to clear the field for conventional wheat sowing [26]. Typically, WH is composed of non-starch polysaccharides (NSP, 41–60%), starch (10–20%), and protein (15–20%) [27]. Presently, it is primarily used as animal feed, but it also serves as an excellent substrate for microorganisms. Using dilute sulfuric acid for pretreatment, the NSP content, primarily hemicellulose, is efficiently converted into monosaccharides, while the starch is hydrolyzed into glucose. Additionally, the trace elements and amino acids released during the hydrolysis of wheat brand provide suitable nutrients for microbial growth.

During the fermentation process, various by-products are also formed like butyric acid and 1,3-propandiaol. In case of butyric acid, by changing the metabolic pathway allows conversion into butanol. The conversion of butyric acid to butanol is a relatively short process, both metabolically and in terms of energy. The standard Gibbs free energy of formation at 25°C and 101.3 kPa for butyric acid ($\Delta fG° = -352.63$ kJ mol^{-1}) is quite close to that of butanol ($\Delta fG° = -171.84$ kJ mol^{-1}), especially when compared to the substrate glycerol ($\Delta fG° = -488.52$ kJ mol^{-1}). Therefore, efficiently absorbing and converting butyric acid will directly enhance butanol production [28]. Previously, numerous studies have been done on variation in reaction conditions like temperature, pH, pressure, substrate, nutrition composition of the medium etc. but a limited number of studies have been conducted on effect of butyric acid on butanol production [29, 30]. This study includes butyric acid production with bacterial hydrolysis and then its conversion into butanol to know the effect of butyric acid on overall butanol production.

Materials and Experimental Procedures

WH was collected from agricultural waste in Charkhi Dadri, Haryana, India. All the used chemicals were purchased from Sigma-Aldrich and of analytical grade; bacterial culture was supplied by MTCC Chandigarh.

Proximate and Compositional Analysis of Raw WH

Before processing for butanol fermentation wheat bran samples should be analyzed for proximate and compositional analysis. Proximate analysis route provides the composition of biomass and its sustainability as a fuel. The chemical composition of biomass, typically focusing on elemental constituent was determined with the help of compositional analyses [31, 32].

Moisture Content

The moisture content of the WH is the amount of water present expressed as a total weight of sample. The moisture content of WH was determined by using a hot air oven. 100 grams of wheat bran was put into the oven at 80°C for 6–8 hours until the mass varied <0.1 g. The reduced weight by 100 (g/g) was the moisture content of the wheat bran.

High moisture content can decrease the energy content of biomass and affects its energy efficiency. It was measured using the drying method. This process involved drying at a temperature of 80 ± 2℃ for duration of 3 hours. Then it was calculated by the equation.

$$\text{Moisture content} = \frac{\text{Wi}-\text{Wf}}{\text{Wi}} \times 100\% \tag{1}$$

where, W_i is initial mass before drying and W_f is the final mass after drying.

Ash Content

Ash content is the non-combustible residue present in biomass which is mainly inorganic materials. Increasing ash content is directly related to calorific value (heat value) of biomass because ash does not contribute to combustion. The ash content was determined following the ASTM D 2017 (1998). A 3 g of husk sample was weighed and placed in a pre-weighed crucible, then incinerated in a muffle furnace at 550℃ until it was completely ashed (approximately 2 hours). The crucible was then moved to desiccators for cooling and ash content calculated by

$$\text{Ash content} = \frac{\text{Wa} - \text{Wc}}{\text{Wb} - \text{Wc}} \times 100\% \tag{2}$$

where, W_a is the weight of ash obtained + crucible weight and W_b is the weight of WH before ash formation + crucible weight and W_c is the weight of the empty crucible.

Volatile Matter

Volatile matter consists of various chemicals in biomasses that are released as gases or vapors when the biomass is heated to high temperatures in the absence of air. This includes a wide range of compounds, such as water vapor, methane, hydrogen, carbon monoxide, and organic compounds like tars and oils. The volatile matter content is a crucial indicator of how quickly and easily biomass will ignite and burn. Biomass with high volatile matter content tends to ignite more easily and burn more rapidly, which is particularly important in processes like gasification and combustion. The volatile matter content was determined following the ASTM, E-872. A 2 g of the sample of the WH was weighed and incinerated in a muffle furnace at 600°C for 30 minutes, and then moved to cool down in a desiccator. Volatile matter content was calculated using the equation.

$$\text{Volatile content} = \frac{\text{Wi}-\text{Wf}}{\text{Wi}} \times 100\% \tag{3}$$

where, W_i and W_f are, respectively, initial and final weight of the sample. The fixed carbon content of the husk was determined by following the ASTM D3172 – 13 as given in Equation (4).

Fixed Carbon Content

Carbon remains in the biomass after volatile materials are released during heating are known fixed carbon content. The fixed carbon content of biomass impacts how the biomass burns and its combustion temperature. With increasing fixed carbon content means biomass can sustain combustion for longer periods. It will be calculated using Equation (4).

$$\text{Fixed carbon content} = 100 - (\text{Ash content} + \text{Volatile matter content}) \times 100\% \tag{4}$$

Extractives

The water soluble and ethanol soluble extractives were determined using the procedure as previously described. For the water extractives, 10 g of WH was taken into 200 ml of distilled water and heated at 80°C for 3 hours and then cooled. After cooling the mixture then dried at 80°C in a hot air oven until weight remains constant. For ethanol extractives, 5% ethanol solution was prepared water extractives added. Then, the solution was kept on water bath for 3 hours at 80°C, cooled, filtered, and dried. The total extractives were calculated as,

$$\text{Extractives} = \frac{\text{Mi}-\text{Mf}}{\text{Mi}} \times 100\% \tag{5}$$

where M_i and M_f represent the initial and final mass of the sample.

Total hemicellulose content was measured by following the procedure outlined in ASTM D5896-96 (2012). One gram of the dried, extractive-free sample was used. To this, 10 ml of NaOH (0.5 M) solution added and then heated at 80°C for three hours in a boiling water bath. Afterward, the residue was filtered and rinsed with distilled water to obtain neutral pH. Dried residues used to calculate hemicellulose content using the specified Eqaution (6), where M_i represents the initial mass of the sample and M_f represents the final mass after treatment.

$$\text{Hemicellulose content} = W_i - \text{W}_f \tag{6}$$

where W_i and W_f represents the weight of extractives free sample taken and final weight after treatment.

Lignin content was calculated according to the procedure given in the institute of paper chemistry, Appleton, Wisconsin as described in [33]. Approximately 2 grams of the extractive sample were treated with 20 ml of 98% H_2SO_4. An acidic solution was added drop by drop to the sample while stirring continuously. Subsequently, transferred to a round-bottom flask and diluted with 3% acidic solution. It was then boiled for four hours. The lignin was filtered and washed with hot distilled water until neutral pH obtained. The filtered lignin sample was then dried at 105°C for 6 to 8 hours and its quantity estimated following the provided equation.

$$\text{Lignin content} = \frac{W_d}{W_i} \times 100\% \tag{7}$$

$$0.\ \text{content} = 100 - (\text{hemicellulose} + \text{lignin} + \text{extractives})\ \% \tag{8}$$

WH Pretreatment

100 g of WH (moisture content, 9.38% w/w) was soaked into dignifying solution (glacial acetic acid (2.4 v/v%), sodium acetate trihydrate (5.4 wt./v%) and sodium chlorite (2.8 wt./v%)) for 18 hours with agitation at 300 rpm and them washed multiple times with distilled water for unreacted chemicals removal [34]. After washing the sample was dried and treated with acidic solution for 1 hour to remove metallic impurities and to alter the structural integrity. Then the pH of the solution was adjusted to 6.5 with $Ca(OH)_2$ at 50°C for 2 hours with agitation at 300 rpm. The $Ca(OH)_2$ was used for neutralization and detoxification of wheat husk hydrolysate. The mixture was then centrifuged at 10,000 rpm for 15 min. Some parts of this liquid were neutralized and diluted with ultrapure water at ratio 1:3 and used to estimate sugars. The clear supernatant, sulfuric acid treated WH hydrolysate was used as carbon source in the following fermentation studies.

Two-Step n-butanol Production from Pretreated WH

Microorganism Culture Preparation

Freeze-stored bacteria *Staphylococcus sciuri* MTCC 6154 were activated in nutrient broth (NB) media containing peptone (5 g/L), beef extract (3 g/L), NaCl (5 g/L) and agar (15 g/L) dissolved in distilled water and sterilize by autoclave. 100 µl of growing culture was spread on agar media plates with serial dilution for CFUs (Counting colony forming units).

Conversion of Biomass Sugars into Fermentable Sugars

As we know, acid hydrolysis breaks down biomass structure and makes cellulose and hemicellulose more accessible. The effectiveness of this treatment influences the extent to which *Bacillus subtilis* can access and hydrolyze biomass. *Bacillus subtilis* can produce cellulases and hemi-cellulases naturally, which can hydrolyze cellulose and hemicellulose into fermentable sugars, such as glucose, xylose and other oligosaccharides and then ferment these sugars into organic acids, such as butyric acid and pentatonic acid. A specific amount of acid hydrolyzed WH maintained pH 6.5 (pH maintained with $Ca(OH)_2$) was placed with 100 µl of bacterial culture at temperature 37°C for 48 hours in an incubator. After 48 hours, the samples were centrifuged for 10 min at 10,000 rpm, and the supernatant was collected for a total analysis of organic acids like acetic acid, butyric acid, pentatonic acid, etc.

WH Synthesized Butyric Acid Fermentation with Bacterial Culture for Butanol Production

After 48 hours of incubation of the first stage, the bottles were removed from incubation and prepared for the second stage. The pH of the broth after n-butyric acid production decreases below 5. To make the pH conditions congenial for the growth of the second stage culture, it was necessary to raise pH to 6.5 by use of 5N NaOH. To extract oxygen, nitrogen was purged into the culture bottle to maintain anaerobic conditions for microorganisms. Then, 100µL of actively growing culture was inoculated into the bottles. Then the bottles are placed in an anaerobic container, instead held at 37°C for 72 hours in the incubator. The fermentation samples were taken at 24-hour intervals. After 72 hours, samples were taken and centrifuged at 10,000 rpm. Clear fluid was processed for ABE analysis.

Experimental Analysis

Gas Chromatography Mass Spectrometry analyzed fermentation products (Acetone, Butanol, and Ethanol) using a packed column. Samples were taken from the solvent phase during fermentation, filtered, and immediately analyzed on a GCMS-QP2010 Ultra (Shimadzu, Japan) system equipped with a DB-5 ms column (30 m length, 0.25 mm inside diameter, 0.25 µm thickness, Agilent, USA). The flow rate of the helium carrier gas was 1 mL /min. The interface and ion source temperatures were set to 250 and 200 °C, respectively. The electron impact voltage was set to 70 eV. The *m/z* range was 35–500. The column temperature was initially set to 100 °C, after which it was increased to 250°C at a rate of 20 °C/min, where it was held for 5 min. Sugars have been calculated using the DNS method and optical density (OD) have been checked at 530 nm, a standard curve has been prepared and sample concentration value has been obtained by a standard glucose curve. A method of optical density (540 nm) was used to measure cell concentration and is described as dry weight cell concentration. Fourier Transform Infrared (FT-IR) spectrophotometer (THERMO Electron Scientific Instruments LLC, Nicolet- iS5) was used to find the functional groups present in the sample. The spectral data were collected in transmittance mode with an accumulation of 128 scans at a resolution of 4.00cm$_{-1}$ from 600 cm$_{-1}$ to 4000 cm$_{-1}$ range.

RESULTS AND DISCUSSION

Proximate and Compositional Analysis

Before cellulose extraction, it is important to know the proximate and compositional analysis of WH to understand the suitability of WH for the targeted application. The cellulose, hemicellulose, and lignin content of WH played a great role in yielding the final product. The cellulose content in WH determined was 45.86%. The moisture content, ash content, volatile matter part, and fixed carbon content were, respectively, 9.38%, 10.14%, 73.17% and 16.69%. The extractives, lignin, hemicellulose, and cellulose content were found to be 8.3%, 27%, 18.84% and 45.86%, respectively. There are some metal impurities like Na, Si, K etc. present in the WH sample (Table 1). The bioactive compunds present in the raw WH are shown in the Table 2.

After acid proximate heading hydrolysis, the total amount of reducing sugars present in hydrolysate of WH was 47.9 g/l. Amount of reducing sugars in wheat or rice husks depends on various parameters like temperature condition, fermentation time and acid concentration etc. Similar studies have shown that the maximum production of total reducing sugars present in WH obtained with enzymatic hydrolysis was 116.93 mg/g [35]. The effect of enzymatic hydrolysis on the total reducing sugars is shown in Table 1.

Table 1. Proximate analysis of raw WH.

Proximate analysis	**Parameters**	**Result Value (%)**
	Moisture content	9.38
	Ash content	10.14
	Volatile matter	73.17
	Fixed carbon content	16.19

Table 2. Compositional analysis of raw WH.

Compositional analysis	**Parameters**	**Result Value (%)**
	Extractives	4.058
	Hemicellulose	18.84
	Lignin	27.0
	Cellulose	45.86
	Others	4.242

Table 3. Effect of enzymatic hydrolysis on the total reducing sugars with optimization in parameters.

Substrate	**Hydrolysis Time (Minutes)**	**Acid Concentration**	**Temperature Conditions**	**Total Reducing Sugars**	**References**
Teff straw	60 min	4 v/v%	120℃	25.5mg/g	[36]
	60 min	0.5 v/v%	80℃	15.1 mg/g	
	70 min	4 v/v%	120℃	24.8 mg/g	
Rice husk	5 min	–	210℃	14.44 g/L	[37]
	15 min	–	230℃	3.19 g/L	
Wheat bran	30 min	2.66 v/v%	121℃	72.7 g/L	[38]
Rice bran	16 min	1.58 v/v%	130℃	89.4 g/L	

Table 3 shows that the amount of total reducing sugars is dependent on acid concentration and hydrolysis time used in hydrolysis. The amount of total reducing sugars increases with increase in acid concentration. This happens may be due to high temperature and acid amount, which leads to more hydrolysis of hemicellulose and amorphous cellulose part. The high concentration of acid is also responsible for lignin removal, which makes the easy exposure of cellulosic part for bacterial or enzymatic hydrolysis.

Fermentation Study of WH for n-Butanol Production

First the inactive bacterial strains were revived in nutrient broth for 12 hours (Figures 2A and B) and then plated on agar media plates for colony count with serial dilution.

Figure 2. Bacterial culture in the NB medium for activation. A: Initiation of activation, B: Activated culture.

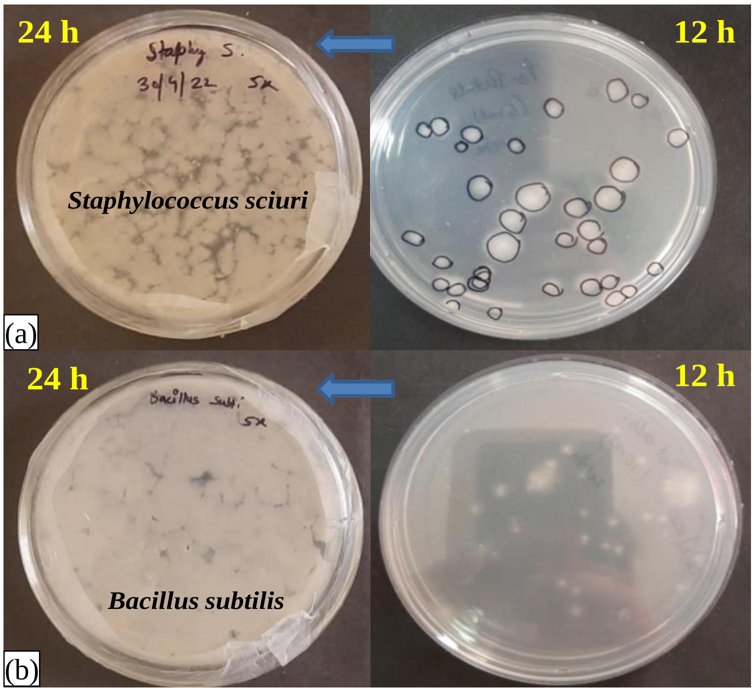

Figure 3. Activated bacterial culture plated on the solid agar media plates at 12h and 24h durations. A: *Staphylococcus sciuri*; B: *Bacillus subtilis.*

The activated bacterial cultures plated on the solid agar media plates at 12h and 24h durations for colony count with serial dilution are shown in Figures 3A and B.

Butyric Acid Production with *Bacillus subtilis*

After 48 hours of incubation of acid hydrolysate of WH, the sample was centrifuged at 10,000 rpm at –4°C for 10 minutes and the supernatant was analyzed with GCMS for butyric acid. The GCMS analysis of each sample was carried out on Thermo Scientific Triple quadruple GC-MS (trace 1300 GC, Tsq 8000 triple quadrupole MS) equipped with a TG 5 MS (30 m * 0.25 mm, 0.25 m) column, Helium was employed as a carrier gas with a flow rate of 1 µl/min and a volume of 2.0 µl. The injector temperature was kept at 250°C while the ion source temperature was fixed at 230°C. The oven temperature was held at 50°C. Butyric acid detected was verified based on relative retention time, peak area percentage, compound name and molecular formula. To get quantitative results, a standard curve was drawn by injecting six different concentrations of butyric acid standards during analysis by GCMS. The retention time of butyric acid is depicted in Figure 4. According to the chromatogram the retention time at the applied conditions was 3.39 minutes. Some other acids like pentanoic acid and hexanoic acid also formed, but the probability of their formation is very less like 11% or 2.3%. Various other by-products are also formed during the process, among them some are furfural, 3-furaldehyde, etc.

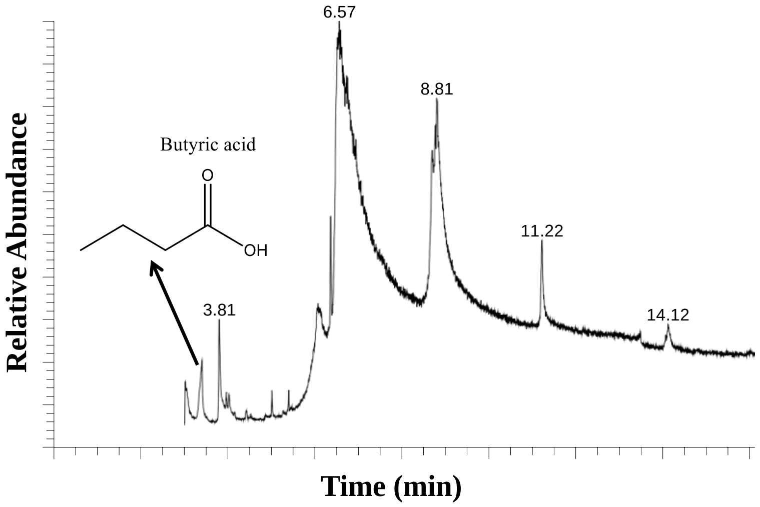

Figure 4. GCMS chromatogram for butyric acid after bacterial hydrolysis.

According to the GCMS chromatogram the probability of butyric acid formation was 82.85% with peak area 18.76% at retention time 3.39 min. The butyric acid production 18.6 µg/20 g was noted with 100µl inoculum volume of *Bacillus subtilis*. In comparison, with an inoculum volume of 3 mL of *Clostridium tyrobutyricum DSM 2637*, a butyric acid production of 4.87 mg per 100 g was recorded by Tasleem Akhtar et al. in 2018. Dwidar et al. (2013) achieved a maximum butyrate concentration of 3.4 g/L from glucose using a fed-batch fermentation process in a reactor with a 5% inoculum of *Clostridium tyrobutyricum* [39, 40]. On the other hand, Li et al. (2020) reported a butyrate yield of 3.87 g/L when 50 mL of fresh cell suspension (inoculum volume) was introduced into each reactor for a comparative study between the wild-type and mutant strains of *Clostridium tyrobutyricum* [41].

Solvent Tolerance of *Staphylococcus Sciuri*

Cell proliferation checked with UV spectroscopy at 600 nm shows rise between 12 to 16 hours, reaching a peak at 48 hours, marking the onset of the stationary phase. By 72 hours, growth nearly stopped. The period between 24 to 48 hours cell proliferation rises vigorously. As we know according to Beer lambert law, the absorbance of a sample is directly proportional to concentration. From the data obtained we can observe that the bacterial growth increases in the sample till 48 hours and after 48 hours it almost became constant. This may be due to an increase in concentration of butanol in the solution, and toxic nature of butanol can disrupt the bacteria cell. The other reason may be insufficient conversion of butyric acid or formation of some other acids in the solution, which may cause decrease in overall pH of the solution, hence inhibiting overall bacterial growth (Figure 5).

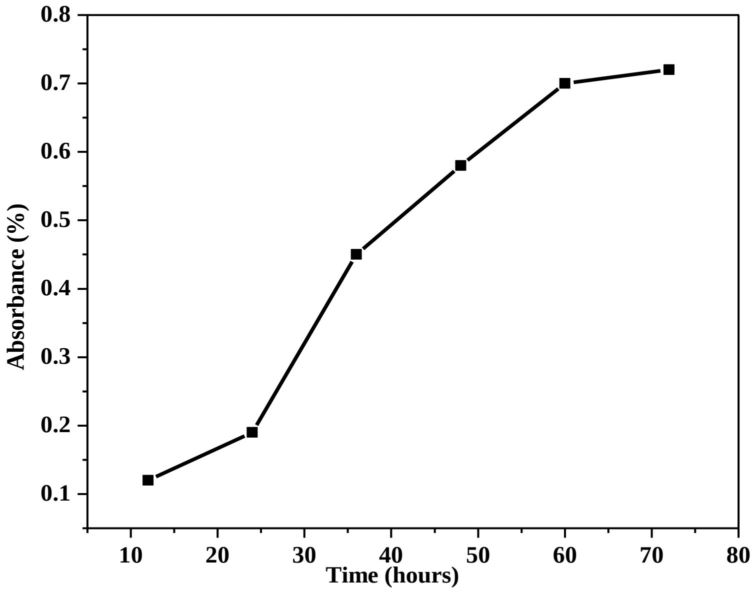

Figure 5. Microbial cell growth.

FTIR Study for Butanol

The FTIR spectra show a broad hump from 3300 cm^{-1} to 3500 cm^{-1}, this shifting in –OH band from 3200 cm^{-1} may be observed due to strong hydrogen bond presence. Some small peaks are observed around 2700 cm^{-1} to 2800 cm^{-1} which is due to the presence of C-H group of CH_3, CH_2 and CH group of butanol and ethanol. Peaks around 1050 cm^{-1} to 1150 cm^{-1} are inductive of C–O stretching vibration of alcohol group [85]. There are some other small peaks also observed in FTIR data which may be due to other impurities present in the sample. The FTIR data did not give us clear assurance of butanol formation so further studies needed for the conformation (Figure 6).

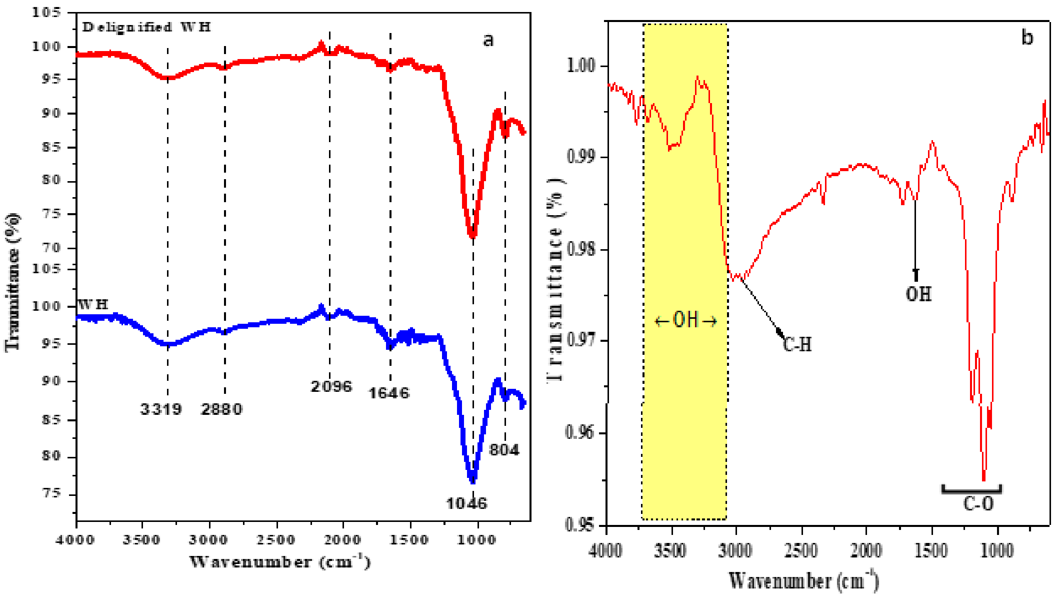

Figure 6. FTIR spectra for (a) WH & delignified WH (b) n-butanol.

GCMS Study for Butanol

A calibration curve was plotted with known concentration of standard n-butanol solution. The chromatogram shown in Figure 7 indicates presence of n-butanol peaks with retention time 3.83. The concentration of n-butanol in the solution was 5.09 µg. At retention time 11.2 peak of 2-hexadecanol in our GC-MS results indicate that the sequential fermentation involving *Bacillus subtilis* and *Staphylococcus sciuri* has led to the biosynthesis and subsequent reduction of fatty acids into fatty alcohols. This multi-step bioconversion involves the initial breakdown of complex carbohydrates into simpler sugars, followed by their conversion into fatty acids and their reduction into alcohol by fermenting microorganisms. The production of ethanol, 2-hexadecanol, and low levels of butanol suggests that *Staphylococcus sciuri* might have multiple metabolic pathways active under our fermentation conditions. Optimizing the conditions, ensuring culture purity, and possibly employing metabolic engineering could help improve the efficiency of butanol production and reduce by-products.

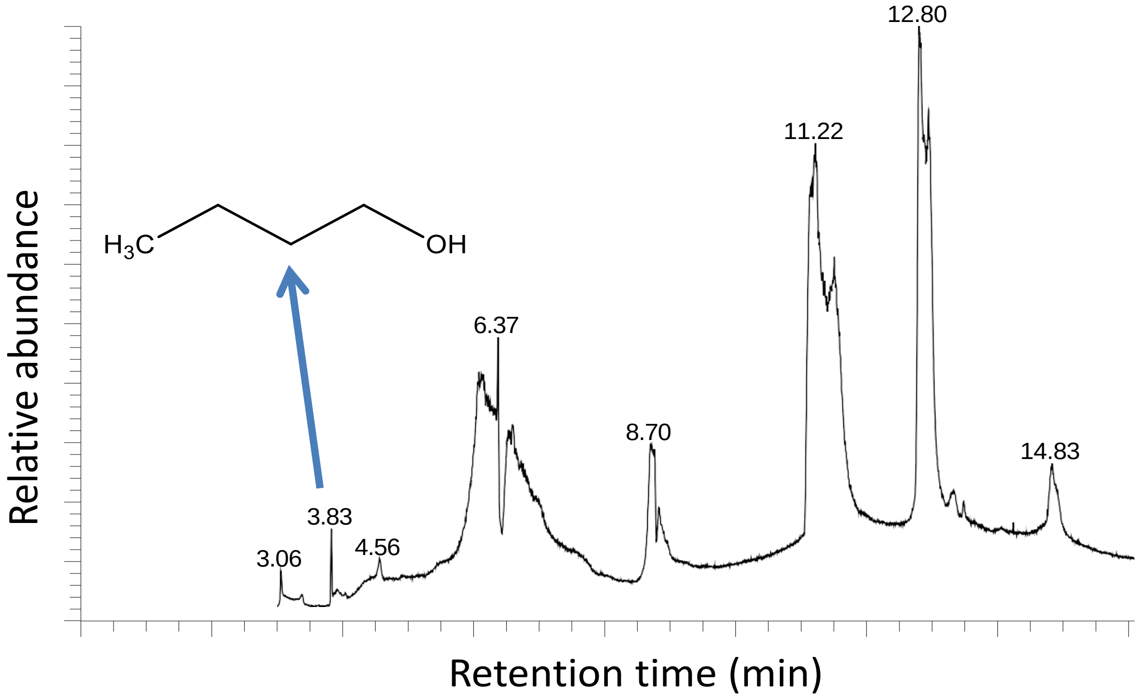

Figure 7. GCMS chromatogram for n-butanol.

CONCLUSIONS

In this study, wheat husk was successfully utilized as a sustainable feedstock to produce n-butanol through a two-step bioconversion process. Initially, wheat husk was converted into butyric acid, yielding 18.6 µg per 20 g of wheat husk. This butyric acid was then further processed to obtain n-butanol, with a final yield of 5.09 µg. GCMS gives approximate concentration of products further testing with HPLC needed to confirm yield. The results demonstrate the feasibility of using agricultural waste, such as wheat husk, as a renewable resource to produce bio-based chemicals, contributing to more sustainable and eco-friendly industrial processes. This approach not only provides a method for valorizing agricultural residues but also supports the growing demand for bio-based n-butanol, which serves as a potential alternative to petroleum-derived chemicals. Further optimization of the conversion process and scale-up studies could enhance product yields, making this method more economically viable for industrial applications.

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Availability of Data and Materials

Not applicable.

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