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Production of biobutanol using Clostridia *Spp* through novel ABE continuous fermentation of selected waste streams and industrial by-products

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ABSTRACT

Contemporary issues such as the unsteady prices, economic and environmental costs of petrol as well as various government policies and efforts on increasing the share of biofuels utilised for energy provision have necessitated the development of fuels from alternative clean, abundant and affordable renewable resources. Among the important biofuels gaining significant prominence in recent years is biobutanol. In as much as there are studies on production of biobutanol from wastes, there is paucity of information on biobutanol production from wastes such as wood hydrolysate, pot ale and laboratory hand towel using acetone-butanol-ethanol (ABE) continuous fermentation process. This study focused on obtaining more information on this through an eco-friendly strategy for renewable, thus bridging the gap in knowledge. This work investigated biobutanol production from selected waste streams and industrial by-products (wood hydrolysate, pot ale and laboratory hand towel) using selected solventogenic Clostridia strains through ABE continuous fermentation process. Wood hydrolysate fermentation broth with C. *saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) produced a butanol concentration of (2.49 g/L) making up 88.3% of the total solvent concentration. Supplementation with two-fold diluted pot ale yielded a total solvent concentration of 3.66 g/L with butanol representing over 84.1%. Maximum acetone-butanol-ethanol solvents production was obtained when supplementation with two-fold concentrated trypton-yeast extract-acetate medium (TYA) was utilised with total solvent concentration of 9.37 g/L and butanol making up 65.5%. Simultaneous saccharification and fermentation of used laboratory hand towel (LHT) yielded a total solvent concentration of 4.78 g/L with C. *saccharoperbutylacetonicum*.

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Introduction

Over the years, the world has been faced with diverse challenges associated with the use of fossil fuels. The constant depletion of fossil fuels, which we are heavily dependent upon, and the uncertainty posed by the volatility of crude oil prices have been extensively reported [1–5]. Moreover, studies have further corroborated the declining rate of global oil production amounting to a 4–5% fall, coupled with an increase in world energy demand [6,7]. Similar claims put the lifetime of the world's oil reserves to be at least 44 years without considering unexplored mineral deposits which may extend this for another 20–40 years [8]. This once more underscores the limited nature of fossil fuels. Furthermore, the negative environmental impacts associated with the exploration and utilisation of fossil fuels provide a great motivation to search for an alternative. This has led to the increasing attention currently accorded the development of biofuels from biomass; which in part is due to the fact that this energy resource is easily replenished, abundant and practically unlimited in availability, and stems from naturally occurring green plants capable of carrying out photosynthesis and contributing to net neutral carbon emissions [8].

Biofuels production from biomass has suffered major setbacks through the years due to the perceived impact it may have on the competition between the use of organic substances as food and their use as fuels by humans. This production from food crops limits their sustainability [9]. These and more have therefore created a need to develop and/or provide alternatives to obtaining biomass feedstock for the production of biofuels from sources that would in no way affect the human food chain; and has led to a significant impetus for research and development on the viability of selected low-value waste streams and industrial by-products recognised as inexpensive fermentative feedstock for the production of biofuels using a wide variety of industrial processes.

Although there are various studies on biobutanol production from wastes, none of these studies applied the method as well the kind of wastes and concentrations used in this current study (10–15). For example, Plaza et al. (2017) studied biobutanol production from brewer's spent grain hydrolysates by Clostridium beijerinckii DSM 6422 [10]; Amiri & Karimi (2015) studied improvement of acetone, butanol, and ethanol production from woody biomass using organosolv pretreatment [12]; Li et al. (2019) worked on n-Butanol production from lignocellulosic biomass hydrolysates without detoxification by Clostridium tyrobutyricum in a fibrous-bed bioreactor [14]; Guan et al. (2017) studied hemicellulose prehydrolysate as a liquid sugar source for butanol production using ABE with hot-water treatment as extraction mean [11]. The list can go on and on. Sequel to literature search, most investigations either used a different process, microbes or waste stream which is different from that of the current study.

Among these low-value waste streams is wood hydrolysate, laboratory hand towel and pot ale. Wood hydrolysates represent considerable wastes generated from wood processing enough to cause environmental harm if not properly managed. However, this waste contains significant amounts of various fermentable sugars thereby justifying its potential use as a substrate for the production of biobutanol, a highly attractive biofuel that has grown from the chemical industry where it is also used as a precursor for polymers and plastics [15]. It has further been reported that wood hydrolysates are rich in organic matter consisting of starch with non-starch polysaccharide materials [1]. Pot ale constitutes another regionally abundant industrial by-product obtained from whisky production. Studies have shown that pot ale which contains low total solids is made up of dead yeast cells, yeast residues, soluble proteins, soluble nutrients, carbohydrates as well as other materials arising from the fermentation and mashing processes involved in the production of malt whisky [16]. Pot ale al so provides an alternative nutritional source for solvent producing organisms such as Clostridia which are capable of fermenting these nutrient sources into useful industrial solvents such as acetone, butanol and ethanol in the ABE fermentation process [1]. Waste paper towels, in many countries of the world, are either recycled, incinerated or end up in landfill sites [17,18]. However, the economic value of waste paper towels can be determined when converted to liquid or gas fuel (ethanol or hydrogen) in fermentation [13]. Acid hydrolysis of waste papers has been found to provide sufficient glucose concentration for fermentation [17,18].

Acetone-butanol-ethanol fermentation in the past has experienced a dwindling industrial application due to stiff competition from the petrochemical industries that are able to obtain cheap raw materials for the production of important solvents. The rise in prices of these raw materials also led to a shift of focus away from these industries with more attention paid to alternatives such as readily available and affordable biowastes with potential for biofuel production. Conventional ABE fermentation process utilizes starchy feedstocks for instance corn and the likes. In 2008, the price of corn was around 1550 Yuan per ton [19]. The price of corn was reported to be around 2000 Yuan per ton in China in the first quarter of 2011 [20]. Similar increment was reported for other feedstocks such as cassava, yam, molasses, and the likes [19,20]. Waste streams such as wood hydrolysates have therefore been recognised as one of those alternative cheap raw materials with the potential for the production of biofuels that are able to sustain the economic balance of the ABE fermentation [1]. In addition to these, other industrial and domestic organic materials commonly regarded as wastes are currently being investigated for their potential as biomass substrates for ABE fermentation. Among these are pot ale and used laboratory hand towel (LHT) which may offer sustainable and affordable routes to obtaining clean and eco-friendly biofuel.

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Several bacteria species belonging to the genus Clostridia have been identified with the capacity to utilise or degrade a wide array of biomass into simple carbohydrate units such as glucose, fructose, sucrose and maltose which can be subsequently converted into biofuels such as biobutanol and other useful chemical/solvents like acetone and butanol. The diversion of carbon rich biomass from landfill reduces the overall carbon footprint of the entire process while providing a readily available and affordable fermentation feedstock for biofuel production. On the other hand, it is important to evaluate the ability of Clostridia *Spp* to produce biobutanol as a primary metabolite, in order to design a robust and efficient novel ABE fermentation process for biofuel production. Thus, various process factors and conditions can in principle affect the production of biobutanol from these wastes such as source of the substrate, concentration of the substrate, and type of pure culture - the Clostridia *Spps*. Although a number of studies have been published on the effect of these process conditions and factors on biobutanol production [21,22]; considering the large number of such waste streams and industrial by-products as well as a wide range of process conditions that can affect their degradation, the literature evidence on this topic is still limited and there is need for further experimental study.

In this study ABE solvents (acetone, butanol and ethanol) producing capacities of five strains of Clostridia *Spp* (C. *acetobutylicum* ATCC824, C. *saccharolyticum* NCP262, C. *beijerinckii* NCIMB 8052, *C. saccharoper-butylacetonicum* N1–4 (NCIMB 12,606) and C. *beijerinckii* BA101) were investigated using various fermentation substrates which include wood hydrolysate, pot ale and used laboratory hand towel. This study considered the effect of process conditions and parameters (kind of substrate, substrate concentration, type of microbial pure culture species, medium composition) on biobutanol production, with the ultimate aim of improving the understanding of their biodegradation and identifying which process conditions should be used to maximise the biofuel production process.

Materials and methods

Bacteria strains and culture maintenance

Bacteria strains *C. acetobutylicum* ATCC824, *C. saccharolyticum* NCP262, *C. beijerinckii* NCIMB 8052, *C. saccharoperbutylacetonicum* (N1–4) NCIMB 12,606 and *C. beijerinckii* BA101 used for this research were obtained from a culture collection of the Biofuel Research Centre, Edinburgh Scotland United Kingdom. Spores of these strains were routinely maintained as spore suspensions in sterile distilled water at 6 °C. To revive the spores to their vegetative state, 1 ml of each strain was heat shocked using a water bath at 80 °C for 10 min and inoculated into 15 ml reinforced Clostridia medium (RCM) contained in a 20 ml universal screw-capped bottle anaerobised overnight to provide the starter culture, followed by overnight incubation at a temperature of 34 °C under conditions of N_2-H_2-CO_2 (80:10:10) gas mix in a Don Whitley Anaerobic work station (Whitley DG500).

Subculture of bacteria strains

The overnight broth cultures (starter cultures) of the strains observed to be actively growing were sub-cultured. Starter culture (1 ml) was inoculated into 15 ml each of trypton-yeast extract-acetate (TYA) medium and the relevant sugar mixture (glucose, fructose and xylose) followed by overnight incubation at 34 °C under conditions of N_2-H_2-CO_2 (80:10:10) gas mix in a Don Whitley Anaerobic work station (Whitley DG500).

Preparation of growth media

All bacteria nutrient media except otherwise stated were obtained from Oxoid Ltd, Basingstoke, Hampshire, England and were prepared according to manufacturer's instructions.

Reinforced clostridia medium (RCM)

RCM was prepared by dissolving 38 g of the dehydrated RCM powder in 1 L of distilled water, agitated with a magnetic stirrer, and heated till a uniform solution was obtained. The resulting RCM solution was allowed to cool and 15 ml volume aliquoted into universal bottles, autoclaved at 121 °C for 15 min.

Reinforced Clostridia Agar (RCA)

Reinforced Clostridia Agar was a solidified version of RCM used for the enumeration of anaerobes using pour plate, shake tube or membrane filtration methods.

The RCA was prepared by dissolving 52.5 g of RCA powder (CM0151) in 1 L of distilled water and agitated with a magnetic stirrer while heating with a Fisher Stirring Hotplate till a clear uniform solution was obtained at boiling temperature. The resulting solution was allowed to cool, split between two 500 ml flasks and sterilised by autoclaving at 121 °C for 15 min.

Trypton-yeast extract-acetate medium (TYA)

The methods of Al-Shorgani et al., 2012 were adopted and modified in preparing the TYA [23]. Quantities of the various compounds listed in the following composition, were dissolved in 1 L of distilled water and heated to boil using Fisher Stirring Hotplate. The TYA contained the following: Yeast extracts, 2g: Tryptone, 6g: Ammonium Acetate, 3g: $MgSO_4.7H_2O$, 0.3g:

KH_2PO_4, 0.5g: $FeSO_4.7H_2O$,0.01 g. The resulting TYA was allowed to cool and aliquoted into 20 ml screw capped universal bottles. All aliquots were sterilised by autoclaving at 121 °C for 15 min.

Fermentation substrates

A compositional analysis of the wood hydrolysate, sourced from a wood processing and digestion facility in Edinburgh, and used in this study shows it contained the following: 4.9 g/L glucose, 55 g/L xylose, 8.7 g/L fructose, 7.7 g/L formic acid, and 0.001 g/L ferulic acid. The pot ale used for the study was obtained from a scotch malt distillery in Edinburgh**.** The laboratory towels used in this research belong to a brand from FSC mixed sources product group (Kimberly-Clark) obtained from recycled wood and/or fibre. The used laboratory hand towels were collected in a special bin from a research laboratory of the Biofuel Research Centre, Edinburgh and were air dried at room temperature. The dried towels were manually shredded and stored on the laboratory bench till required. Shredded hand towels (10 g) were weighed into 250 ml screw capped bottles, followed by the addition of 150 ml of TYA and sterilised by autoclaving at 120 °C for 15 min**.** A control substrate was prepared to monitor and compare the growth and solvent production of the Clostridia strains used in this experiment. The formula for various amounts of sugar was selected to mimic the sugars in the hydrolysate of the selected substrates. Sugar solution used as the control substrate contained in g/l; 4.9 glucose, 55 xylose and 8.7 fructose.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed by adding 600 µl and 100 µ CTec and Htec2 (synthetic Novozymes Cellulase) enzymes respectively to the 10% slurry, while 1200 µl and 200 µl CTec and Htec2 enzymes were similarly added to the 20% slurry of acid hydrolysed LHT respectively. Samples were kept in an incubating shaker at a temperature of 50 °C and a speed of 150 rpm. Samples were collected after 24 h and stored at −20 °C until required.

Fermentation of substrates

Fermentation of wood hydrolysate was carried out in TYA. Wood hydrolysate obtained as previously described and stored at 6 °C was anaerobised until required. Wood hydrolysate (2 ml) was transferred into 8 ml TYA followed by inoculation with 1 ml of each of the actively growing sub-cultured Clostridia strains. The inoculated substrates were incubated at 34 °C for 48 h under conditions of N_2-H_2-CO_2 (80:10:10) gas mix in a Don Whitley Anaerobic workstation (Whitley DG500®).

Fermentation of wood hydrolysate in pot ale

Aliquots (2 ml) of each wood hydrolysate was initially transferred into 8 ml of TYA followed by the addition of 2 ml of 50% pot ale and inoculated with 1 ml of starter culture of the various Clostridia strains. This was incubated overnight at 34 °C hours under conditions of N_2-H_2-CO_2 (80:10:10) gas mix in a Don Whitley Anaerobic workstation (Whitley DG500). Subsequently 2 ml of wood hydrolysate was transferred into 2 ml of pot ale and inoculated with 1 ml of the previously sub-cultured Clostridia strains followed by 48 h incubation at 34 °C under standard anaerobic conditions previously described.

Screening of strains

RCA contained in petri dishes was used as the growth medium for the identification and screening of Clostridia strains. Sterile wire loop was used in streaking inoculum from each selected strain culture onto the RCA plates and incubated overnight at 34 °C under conditions of N_2-H_2-CO_2 (80:10:10) gas mix in a Don Whitley Anaerobic workstation (Whitley DG500). The pH of the broth was monitored before and after inoculation with each of the selected strains as Clostridia strains will favourably grow within a pH range of 4.5 and 7.0. Gram staining was also performed. This was carried out for culture monitoring and species maintenance.

Analytical method

Fermentation broth (1.5 ml) of each substrate was collected into 1.5 ml sterile Eppendorf micro centrifuge tubes and centrifuged at 13,000 rpm for 15 min. The supernatant from each sample was carefully sieved through 0.2 µl, while collecting the supernatant and discarding the pellets. A Chrompack® 9001 GC equipped with CP010 auto sampler and Maestro II, CP SIL 5CB, 10 m length, 0.32 µ diameter column with a split injector and flame ionisation detector (FID) was used to determine the concentration of the ABE solvents. The temperature of the detector and injector were 200 °C and 180 °C respectively. Injection at appropriate temperature as well as detection can of course also be used to improve analyte detectability. At these temperatures, with the detector selectivity sufficiently high, the detection limits will improve proportionally with the volume injected. A solvent peak is obtained with most GC detectors. Helium was used as the carrier gas. Sugar analysis was carried out using high-performance liquid chromatography (HPLC).

For monosaccharide analysis, 2 ml each of the samples was collected using a 2.5 ml syringe and filtered through a 0.2 µm syringe filters into vial tubes. Mesoerythritol (10 µl) was added to each sample as internal standard and vortexed for at least

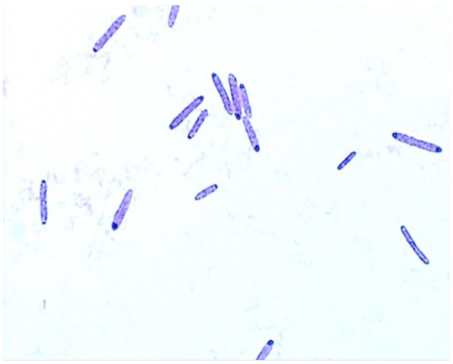

Fig. 1. Gram stains of a culture of *C. saccharolyticum* NCP262 Magnified to 100X.

Table 1
Concentrations of the ABE solvents produced after 48 h of the fermentation of wood hydrolysate and control substrate using *C. saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) and *C. saccharolyticum* NCP262. Initial total sugar concentration was 68.61 g/L.

Strains & substrates	Acetone%	Butanol%	Ethanol%	Acetone g/L	Butanol g/L	Ethanol g/L	ABE g/L
C. saccharoperbutylacetonicum NCIMB 12,606 (N1–4) & wood hydrolysate	0.07	0.24	0.00	0.33	2.49	0.00	2.82
C. saccharolyticum NCP262 & wood hydrolysate	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C. saccharoperbutylacetonicum NCIMB 12,606 (N1–4) & control substrate	0.08	0.08	0.00	0.00	0.65	0.00	0.65
C. saccharolyticum NCP262 & control substrate	0.00	0.04	0.00	0.00	0.31	0.00	0.31

10 s. Samples were analysed using HPLC with calibration standards comprising mesoerythrilose, fructose, maltose and glucose at concentrations varying from 2.5 mg/ml, 5.0 mg/ml and 10 mg/ml each. The HPLC system used for the sugar analysis consisted of a Spectraphysics SP8800® ternary Pump (model sp8800–020), thermo separation product spectra-series AS100 auto sampler and equipped with spectraphysics refractive index detector series sp6040XR RI detector. DataApex Chromatography Station for Windows software (CSW32), version v.1.4 was used as the control/data acquisition software. The column used was a REZEXTM RPM Pb^{2+} monosaccharide column (300 × 7.8 mm) at 80 °C.

Results and discussions

The ability of different Clostridia species to produce biobutanol during the ABE fermentation of selected waste streams (wood hydrolysate, pot ale and used laboratory hand towel) was investigated. The products of substrate hydrolysis and concentrations of acetone, butanol and ethanol produced have been presented as seen in the relevant tables. Values shown represent the average of at least three (3) measurements.

Screening of strains

Routine monitoring of the inoculated cultures for contaminations ensured only pure cultures of the selected strains were utilised for the fermentation. They were also observed for active growth before inoculation into substrates was carried out. The broth culture of each selected strain was also observed for the production of odour, gas and turbidity characteristic of the Clostridia strain. Results from Gram staining performed is represented in Fig. 1.

ABE solvent analysis of wood hydrolysate fermentation by different strains of solvent producing clostridia

Experiments were conducted to study the possibility of obtaining biobutanol from the ABE fermentation of the selected waste streams and industrial products. Wood hydrolysate containing 68.6 g/L total sugars as well as 0.54 g/L hydroxymethylfurfural (HMF), 7.7 g/L formic acid, and 0.001 g/L ferulic acid was used as the fermentation substrate in a media containing TYA and actively growing cells of *C. saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) and C. *saccharolyticum* NCP262. After a fermentation period of 48 h, samples were collected from the fermentation broth and the concentrations of ABE solvents were determined. Results obtained are as shown in Table 1.

Low concentrations of solvents were obtained in the fermentation broth with C. *saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) producing the highest concentration of butanol (2.49 g/L) which makes up about 88.3% of the total fermentation broth. The amount of butanol produced by C. *saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) represented 3.6% of the

Table 2
ABE solvents concentrations obtained from the fermentation of wood hydrolysate supplemented with pot ale by C. *saccharolyticum* NCP262 over a 72-hour period.

Substrates	Acetone (%)	Butanol (%)	Ethanol (%)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)	Final pH
Wood hydrolysate*	0.11	0.22	0.02	0.12	1.83	0.12	2.07	5.03
Control substrates	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.53

* supplemented with pot ale.

Table 3
ABE solvents concentrations obtained from the fermentation of wood hydrolysate supplemented with pot ale by *C. saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) over a 48-hour period.

Substrates	Acetone (%)	Butanol (%)	Ethanol (%)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)	Final pH
Wood hydrolysate*	0.07	0.37	0.01	0.52	3.08	0.06	3.66	6.11
Wood hydrolysate	0.00	0.01	0.01	0.00	0.01	0.05	0.06	5.44
Control substrates	0.05	0.51	0.00	0.36	4.25	0.00	4.61	5.08
Control substrates	0.06	0.61	0.031	0.52	5.06	0.26	5.77	5.20

* supplemented with two-fold diluted pot ale, a two-fold sugar dilution.

total sugars present in the fermentation media (Table 1). Although the observed concentration of butanol was low, however, it exceeded the concentration of butanol produced from the fermentation of the control substrates as well as the amount produced when C. *saccharolyticum* NCP262 was used (Table 1). This suggests a suitability of the former strain on the substrate than the latter. This can be explained by the superior capacity of C. *saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) to reutilize acids produced or supplied during ABE fermentation of a wide range of substrates [21,24].

However, other factors may be attributed to the low solvents production from the fermentation of wood hydrolysate. For instance, the compositional analysis of the hydrolysate showed the presence of chemical compounds such as furfural, hydroxymethylfurfural (HMF), formic acid, ferulic acid some of which are known to possess properties that may be inhibitory and/or stimulatory to the ABE process. Although, HMF and furfural have been reported to have no inhibitory but rather stimulatory effect on solventogenic strains of Clostridia [25], the presence of 7.7 g/L formic acid representing 10% of the entire chemical constituents however, appears significant to suppress the growth of the strains used. Formic acid at a concentration as low as 1 mM or 46 ppm has been postulated to potently inhibit Clostridia inside the cell wall [7].

ABE solvents analysis of a 72-hour fermentation of media containing wood hydrolysate and pot ale by C. saccharolyticum *NCP262*

A fermentation medium containing wood hydrolysate was supplemented with equal volume of diluted pot ale (50%) as a source of nutrient and was inoculated with actively growing cells of C. *saccharolyticum* NCP262, incubated for a period of 72 h. To compare the results obtained, a control substrate was set up as previously described in methods section. Concentrations of the solvents obtained are as shown in Table 2.

The supplementation of wood hydrolysate with pot ale did not appear to have improved the production of ABE solvents in this experiment (Table 2). The results indicate poor ABE solvents production in the fermentation broth, of which butanol makes up over 88% of total solvents. No solvent was obtained with the control substrate and the final pH suggested the production of acetic and butyric acids. However, since the production of neutral solvents was the interest of this investigation, no account of the acids produced were reported.

ABE solvents analysis of a 48-hour fermentation of media containing wood hydrolysate and pot ale by C. saccharoperbutylacetonicum *ncimb 12,606 (N1–4)*

In this experiment, a similar approach taken in the previous Section (3.3), was adopted using an actively growing culture of C. *saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) on fermentation media containing wood hydrolysate and diluted pot ale. The pot ale was further diluted (X 2) with distilled water to observe if its concentration has any inhibitory effect whatsoever. After 48 h of continuous fermentation process, solvents analysis of the fermentation broth showed the various concentrations of detected solvents as presented in Table 3. Values recorded represent the average of at least three measurements.

The fermentation broth containing the two-fold diluted pot ale had a total solvents concentration of 3.56 g/L with butanol representing over 84%. This indicate the implication of further diluting the pot ale used in the fermentation as a significantly higher solvents production was obtained compared to when the pot ale that was not additionally diluted was used (Tables 2,3). The pot ale used in both cases were buffered to a pH of at least 5.5. The presence of several inorganics such as potassium, calcium, magnesium silicates, sulphates and particularly free copper ions have been demonstrated to have an

Table 4
ABE solvents production from the simultaneous saccharification and fermentation of used laboratory hand towel (LHT) by five (5) Clostridia strains (C. saccharolyticum NCP262, C. beijerinckii NCIMB 8052, C. beijerinckii BA101, C. saccharoperbutylacetonicum NCIMB 12,606(N1–4) and C. acetobutylicum ATCC824) and Cellulase enzymes.

Substrate & Strain	Acetone (%)	Butanol (%)	Ethanol (%)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)	Final pH
C. saccharolyticum NCP262 + LHT	0.07	0.18	0.00	0.36	1.48	0.00	1.84	4.83
C. beijerinckii NCIMB 8052 + LHT	0.01	0.08	0.00	0.11	0.63	0.00	0.75	5.03
C. beijerinckii BA101 + LHT	0.01	0.13	0.00	0.11	1.08	0.00	1.19	5.12
C. saccharoperbutylacetonicum NCIMB 12,606(N1–4) + LHT	0.15	0.43	0.00	1.22	3.56	0.00	4.78	5.58
C. acetobutylicum ATCC824 + LHT	0.05	0.25	0.00	0.41	2.11	0.00	2.51	5.14
C. saccharolyticum NCP262 + Control substrates	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.88
C. beijerinckii NCIMB 8052 + Control substrates	0.00	0.03	0.00	0.00	0.21	0.00	0.21	5.03
C. beijerinckii BA101 + Control substrates	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.27
C. saccharoperbutylacetonicum NCIMB 12,606(N1–4) + Control substrates	0.00	0.03	0.00	0.00	0.21	0.00	0.21	5.33
C. acetobutylicum ATCC824 + Control substrates	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.03

Table 5
Concentrations of ABE solvents produced by the fermentation of wood hydrolysate in a two-fold concentrated TYA media by C. *saccharolyticum* NCP262 after 48 h incubation period.

Substrates	Acetone (%)	Butanol (%)	Ethanol (%)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)
Wood hydrolysate*	0.24	0.38	0.00	3.52	5.85	0.00	9.37
Control substrates	0.03	0.23	0.00	0.46	3.51	0.00	3.96

* supplemented with double strength TYA.

inhibitory effect on solventogenic Clostridia strains at a concentration in excess of 20 µM [18]. This may be correlated to the difference observable in the wood hydrolysate fermentation experiment supplemented with diluted and undiluted pot ale.

Simultaneous saccharification and fermentation of used laboratory hand towel (LHT) using selected solvent producing strains of clostridia

To compare the solvent production from used laboratory hand towel by different solventogenic strains of Clostridia, simultaneous saccharification and fermentation were conducted using untreated laboratory hand towel as substrate and the five selected strains of solventogenic clostridia in the presence of a synthetic enzyme (Novozymes Cellulase). The fermentation media contained 10 g dry mass of the used laboratory hand towel suspended in TYA medium, sub-cultured cells of C. *saccharolyticum* NCP262, C. *beijerinckii* NCIMB 8052, C. *beijerinckii* BA101, C. *saccharoperbutylacetonicum* NCIMB 12,606(N1–4) and C. *acetobutylicum* ATCC824) and cellulase enzymes. Solvents analysis of the fermentation broth was carried out after 48 h of continuous fermentative process and the various solvent concentrations produced by each strain are reported in Table 4.

The data shows an unequal trend in the abilities of the various Clostridia strains to convert unhydrolysed LHT to ABE solvents (Table 4). The highest solvent production was observed in the fermentation broth containing C. *saccharoperbutylacetonicum* NCIMB 12,606(N1–4) with a total solvents concentration of 4.78 g/L followed by C. *acetobutylicum* ATCC824 which produced a total solvent of 2.51 g/L. C. *saccharolyticum* NCP262 gave a total solvent yield of 1.84 g/L while C. *beijerinckii* BA101 and C. *beijerinckii* NCIMB 8052 gave a total solvents concentration of 1.19 g/L and 0.75 g/L respectively (Table 4). The Clostridia strains were hardly solventogenic on the control substrates.

ABE solvents yield of the fermentation of wood hydrolysate in a two-fold concentrated tya media using C. saccharolyticum *NCP262*

A fermentation medium containing a two-fold concentrated TYA with wood hydrolysate as the carbon source and a culture of C. *saccharolyticum* NCP262 was set up over a period of 48 h. Table 5 shows the concentrations of the various solvents obtained.

Maximum production of ABE solvents was obtained using two-fold concentrated TYA with a total solvent concentration of 9.37 g/L with butanol making up 62.5% (Table 5). The concentration of the total ABE solvents in this experiment was the highest obtained from the fermentation of the substrates utilised, suggesting that the supplementation with a two-fold concentrated TYA is a key factor in the ABE fermentation process.

Table 6
Concentrations of carbohydrates obtained from the hydrolysis of Laboratory hand towel (LHT) using sulphuric acid (2%) and selected enzymes.

Samples	Sugars				
	Galactose g/L	Sucrose g/L	Stachyose g/L	Arabinose g/L	Glucose g/L
10% slurry of acid hydrolysed LHT	ND	ND	ND	ND	ND
10% enzymatically hydrolysed LHT	3.19	ND	114.87	43.96	ND
20% slurry of acid hydrolysed LHT	7.41	67.31	ND	ND	ND
20% enzymatically hydrolysed LHT	7.52	ND	ND	29.44	7.86

ND*- Not detected.

Concentrations of various carbohydrates obtained from the hydrolysis of laboratory hand towel (LHT) using sulphuric acid (2%) and selected enzymes

The used LHT was hydrolysed using a combination of mild acid treatment (2% sulphuric acid) at a temperature of less than 126 °C and the addition of synthetic enzymes (Novozymes). A 10% and 20% slurries (biomass: acid) were prepared to compare the most efficient and suitable ratio for optimum hydrolysis of the LHT and the various carbohydrates. Their respective concentrations were recorded as seen in Table 6.

Predominant limiting factors for the ABE fermentation process suggested in these experiments may include inhibition of the Clostridia strains; since the treatment of the selected substrates at high temperature and low pH may have produced furfural compounds due to the degradation of sugars as previously described. These compounds at certain concentrations have been reported to inhibit Clostridia growth [15,18]. The wood hydrolysate substrate used contained merely 0.54 g/L of HMF and furfural acids which may appear insignificant. However, the presence of 7.7 g/L of formic acid may be tangible enough to have an inhibitory effect on the solventogenic strains used. The presence of other unspecific inhibitory agents may not be ruled out as well [26]. In addition to the inhibitory effects of compounds produced during the pre-treatment, inaccessibility due to the degree of crystallinity in the cellulose or similar components such as hemicellulose or lignin may be responsible for the inability of the selected strains to utilise substrates such as the used the laboratory hand towel (Table 6).

The final pH of the fermentation broth in most cases fell within the acidic range suggesting that although the substrates may have supported the growth of the Clostridia strains, they were however insufficient to sustain the shift from the acidogenic to solventogenic phase of the ABE fermentation (Tables 1–5), thereby producing more butyric acid than neutral solvents. The poor solvent production obtained from this work can also be attributed to insufficient sugars or nutrients. Previous studies have suggested that solvents production in fermentation systems was limited by deficiency of sugars and nutrients in the fermentation medium [9,15,24].

Sequel to the results from this study, C. *saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) was the most important strain that influenced the conversion of the waste streams utilised into ABE fermentation products (Tables 1–4). Wood hydrolysate fermentation broth with C. *saccharoperbutylacetonicum* NCIMB 12,606 (N1–4) produced a butanol concentration (2.49 g/L) making up 88.3% of the total solvent concentration (Table 1). Simultaneous saccharification and fermentation of used laboratory hand towel (LHT) yielded a total solvent concentration of 4.78 g/L with C. *saccharoperbutylacetonicum* NCIMB 12,606(N1–4) producing 3.56 g/L of butanol (Table 4). Supplementation with two-fold diluted pot ale had an important effect on total solvents production (3.66 g/L) with butanol representing over 84% (Table 3). The highest acetone-butanol-ethanol solvents production was obtained when supplementation with two-fold concentrated TYA was utilised with total solvent concentration of 9.37 g/L and butanol making up 65.5% (Table 5).

One limitation of this study was the inability to carry out a complete compositional analysis of substrates, thus it may be difficult to affirm the presence or absence of trace minerals and nitrogen source in the substrates, which are important for ABE fermentation. Similarly, the sugar analysis of the substrates hydrolysed using the described pre-treatment methods indicates incomplete enzymatic hydrolysis of cellulose in the lignocellulosic materials which the selected waste streams and industrial by-products contain with several unidentified peaks appearing in the chromatograms of the hydrolysates as presented by the HPLC.

Conclusion

Data obtained from the experiments show that different concentrations of ABE solvents were obtained using continuous fermentation process over a period of 48–72 h. The concentrations of solvents obtained during this study using the different strains of Clostridia suggest that the conversion of wood hydrolysate, pot ale, and used hand towel into acetone-butanol-ethanol fermentation solvents is possible at an optimised scale. This study gives insight into the understanding of biobutanol production from waste streams such as wood hydrolysate, pot ale and used hand towel using acetone-butanol-ethanol fermentation. Further indications on the appropriate choice of process conditions for biofuel production using Clostridia *Spp* through novel acetone-butanol-ethanol continuous fermentation of these waste streams and industrial by-products have been provided by this study.

Declaration of Competing Interest

None.

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