Peculiar Response in the Co-Culture Fermentation of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* for the Production of ABE Solvents

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Abstract: Two bacterial strains (CL11A and CL11D) that are capable of ABE fermentation, identified as *Leuconostoc mesenteroides* and *Weissella cibari,* were isolated from the soil surrounding the roots of bean plants. Another strain (ZM 3A), identified as *Lactobacillus plantarum*, which is capable of purely ethanolic fermentation was isolated from sugarcane. Glucose was used as a standard substrate to investigate the performance of these strains in mono—and co-culture fermentation for ABE production. The performance parameters employed in this study were substrate degradation rates, product and metabolite yields, pH changes and microbial growth rates. Both ABE isolates were capable of producing the three solvents but *Leuconostoc mesenteroides* had a higher specificity for ethanol than *Weissella cibari*. The co-culturing of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* enhanced ethanol production at the expense of both acetone and butanol, and also influenced the final substrate consumption rate and product yield. The experiments indicated the potential of these niche environments for the isolation of ABE-producing microorganisms. This study contributes to the formulation of ideal microbial co-culture and consortia fermentation, which seeks to maximize the yield and production rates of favored products.

Keywords: acetone; butanol; ethanol; bioprospecting; microbiological screening; co-culturing; fermentation

1. Introduction

The replacement of fossil fuels with biofuels is one strategy for global decarbonization, hence mitigating the effects of climate change [1]. The main biofuels, including biodiesel, bioethanol, biomethanol and biobutanol have been successfully produced at an industrial scale to replace or reduce the consumption of their fossil fuel counterparts [2–4]. Technologies for biodiesel and bioethanol have already been established and optimized to enable widespread commercial application. Success stories on this front can be found in the United States of America and Brazil [5]. The acetone-butanol-ethanol (ABE) route, which is widely used for solvents and biofuels production, still faces challenges that reduce the profitability of manufacturing this product. The most significant challenge is that butanol-producing microorganisms are inhibited by the end-product concentration and interference with substrates or metabolites, which causes poor butanol yields or early onset biochemical pathway feedback, which leads to process cessation [6]. Butanol has many applications besides being used in fuel blends. It can be used as a base chemical for other industrial processes. As a fuel, butanol is much better and safer than ethanol because of its high calorific value, it has less flammability risk and its reduced solubility in water [7].

The raw materials for biofuels production are classified into readily convertible (first generation) biomasses derived mostly from cereal-based cash crops including maize, sugarcane, sugar beets and rapeseed. Other raw materials include lignocellulosic feedstocks

from agricultural residues and municipal solid wastes (second generation) and algae-based (third generation) fuels, as well as the most recently included biofuels derived from genetic modification of organisms such as cyanobacteria or the modification of non-edible crops that are cultivated on non-arable land (fourth generation) [8,9]. The classification of the raw materials has implications for the technology selection and microorganisms required to achieve the bioconversion of different biomasses into value added products such as biofuels. Microorganisms are highly specific and enzymatically limited in terms of the substrate that they can convert, the products they produce and the rate of bioconversion [10]. However, microorganisms also tend to adapt to their environments so that over time they develop the ability to consume or biotransform "alien" substrates using various strategies that evolve during long-term exposure [11].

The establishment of niche environments with adapted microorganisms tends to make for superior strains compared to other organisms that have never been exposed to that environment and/or substrates [12]. Since it is claimed that the slow acceptance of ABE fermentation technology is partly attributable to poor microbial performance, which manifest as poor yields, slow kinetics, low resistance to product/substrate inhibition, efforts to search for microbes that have improved on these limitations through adaptation should be intensified [13]. Therefore, this research delved into bioprospecting for microbes from two niche environments for ABE fermentation studies. Previous studies show that certain ABE-producing strains, such as the *Clostridium* species tend to thrive in agricultural soils around the roots of legumes [14]. As such, this became the starting position for the present investigation with the premise being that different soils sourced from different sites and different legumes are expected to harbor species with various adaptions, which are then likely to have different ABE fermentation performance characteristics [15]. In this study, microbial strains for ABE production were isolated from soil on which red specked beans had been grown. Similarly, bioprospecting for ethanolic fermentative microbes was also performed using various sources of sugarcane. Sugarcane is the most prevalent natural source of sucrose; this can be used to indicate ethanolic formation due to glucose metabolism [16]. The isolated strains were then enriched, and their fermentation performance tested using glucose as a substrate. The best performing microorganisms from the different sources were investigated for ethanol and ABE production, the isolates were also tested in a co-culturing set up.

Although, mono-culturing for fermentation purposes is rather popular in most industrial applications, co-culturing studies that showed improved product yields and microbe resilience to inhibition have also been studied with significant outcomes [17–19]. Specifically, for butanol, a 203% increase in butanol yield and a 155% increase in productivity was observed when *Saccharomyces cerevisiae* was added to a *Clostridium* monoculture system. Therefore, ethanol-producing microbes and ABE-producing microbes were co-cultured in this study to investigate their effects on the overall ABE fermentation performance.

2. Materials and Methods

2.1. Preparation of Samples

Both the sugarcane (*Saccharum officinarum*) and red speckled broad beans (*Phaseolus vulgaris*) were bought at a Farm Produce supermarket in Pretoria, South Africa. Test samples of sugarcane juice were spontaneously fermented by relying on autochthonous microorganisms, which were later isolated from the fermented liquor.

Test samples of the roots of speckled broad beans were planted in different soil types taken from various sites around Johannesburg and Pretoria in South Africa. The beans were planted in garden pots at a depth of 9 cm depth and left to germinate and grow for 6 weeks in the horticultural greenhouse of the University of South Africa (UNISA), Florida, Johannesburg.

2.2. Microbiological Isolation and Screening

At least 1 g of soil was collected from different positions surrounding the root of the beans and from different potted plants. The pooled samples were mixed into saline solution and agitated using a Vortex Mixer (Thermo scientific 17TS, LP, Johannesburg, South Africa) intermittently for 5 min at 1800 rpm. Replicate samples were obtained from the pooled soil and used as working samples. About 1 mL of supernatant was collected for immediate transfer to potato dextrose agar and nutrient agar plates and incubated at 30 °C for 72 h under anaerobic conditions. Selected colonies from previous media cultivations were taken through several plate transfers using the streaking technique under the same incubation conditions described previously until axenic monocultures were achieved. These axenic cultures grown on broth media and were each subjected to screening using a method adapted from Comstock et al., to determine acetone production by adding 1 mL of 5% sodium nitroprusside and at least ten drops of 25% ammonium solution until a color change was observed [20]. Only pure cultures that tested positive by showing a violet red color as an indication of acetone were employed in the experiments that followed.

Fermented sugarcane juice was used to inoculate yeast media (Sigma-Aldrich, Johannesburg, South Africa) broth containing antifungal agent (ketoconazole). Media pH was adjusted to 6.0. The mixture was purged prior to anaerobic incubation with N_2 gas for 10 min and incubated for 72 h at 30 °C. Screening of the axenic cultures obtained was done using the method described by Dennis and Young, and using Schiff's reagent to test for the presence of acetaldehyde and a positive pink color [21].

2.3. Molecular Characterization

2.3.1. DNA Extraction and Sequencing of Bacteria Isolates

Genomic DNA was extracted from pure and screened isolates using Quick-DNA™ Miniprep Kit following the manufacturers' instruction manual. The 16 S rDNA sequence (5′ to 3′) amplification primers (16S-27F: AGAGTTTGATCMTGGCTCAG and 16S-1492R: CG-GTTACCTTGTTACGACTT) used the onetaq quick-load 2x master mix. Gel electrophoresis was used to run the amplified 16s rRNA gene and the fragments were extracted from gel using the Zymo clean Gel DNA Recovery Kit. The analysis of the sequences was conducted using the CLC bio workbench version 7.6 and the identities of the sequences were obtained after Blasting on NCBI BLAST [22]. The sequences were submitted to the Banklt NCBI submission tool with accession numbers MN078125-MN078128 and MK968722-MK968728.

2.3.2. Phylogenetic Analysis

The sequence relatives were obtained from NCBI. The sequences were aligned using ClustalW multiple alignment on BioEdit and the multiple sequence alignment program (MAFFT) online version. The neighbor-joining method on MEGAX version 10.0.5 was used to deduce the evolutionary history [23]. A bootstrap test of 1000 replicates was used to estimate the reliability of the phylogenetic tree plot [24]. The maximum composite likelihood method as described by Tamura et al. was used to compute the evolutionary distances [25].

2.4. Fermentation Experiment Setup

For the ethanol fermentation, pure screened cultures of bacteria from sugarcane liquor were enriched in broth preparations of yeast media (Sigma-Aldrich, Johannesburg, South Africa). Isolates were obtained from the previous 72-h growth of microorganisms and transferred to a 1 L flask containing a 950 mL mixture of 10% glucose and yeast media. The vessels containing the mixture were purged with N_2 gas for 10 min before incubation. Batch fermentation was allowed to proceed in a water bath maintained at 30 °C for 12 days [26]. Fermenting samples were taken in triplicate and negative controls were set up for this experiment.

For the ABE fermentation, pure screened cultures of bacteria taken from soil derived from the root of speckled broad beans was enriched in reinforced clostridium broth medium

(Sigma-Aldrich, Johannesburg, South Africa) for 72 h in a 50 mL test tube. The inoculum was transferred into the same broth medium but with an addition of 10% glucose, with the volume of the mixture totaling 950 mL. The vessel containing the mixture was then purged with N_2 gas for 10 min before incubation. Batch fermentation was allowed to proceed in a water bath maintained at 30 °C for 12 days [26]. Fermented samples were taken in triplicate and negative controls were set up for this experiment.

2.4.1. Measurement of Glucose Levels during Fermentation

Glucose conversion and utilization were measured by using a UV-visible spectrophotometer at a wavelength of 315 nm as described by Albalasmeh et al. [27]. Standard solutions were prepared in concentrations of 0.005, 0.5, 1, 2, 4,6,8,10,12,14,16,18 and 20 g of glucose and were mixed separately with 100 mL of distilled water. Exactly 1 mL aliquot of each standard solution was rapidly mixed with 3 mL of concentrated sulfuric acid in a test tube and vortexed for 30 s. The solution was then cooled in ice for 2 min to room temperature. Absorbance was measured at 315 nm using a UV spectrophotometer (Thermo-Scientific GENESYS 10S UV-Vis). A standard calibration curve was obtained by plotting the glucose concentration (g/L) against absorbance. Samples were drawn at 24-h intervals and standard calculations were used to evaluate glucose utilization in the ABE fermentation process and the ethanol-producing bacteria fermentation process [28].

2.4.2. Measurement of Alcohols and Acids during Fermentation

The procedure described by Al-Shorgani et al. was adapted and used to measure the solvents (acetone, ethanol, butanol) and volatile fatty acids (acetic acid and butyric acid), using the gas chromatography technique [29]. A 7890B GC-System from Agilent Technologies was set at a 20:1 split ratio and a 1 microliter sample was tested (after it was filtered using a 0.45μm pore size filter and centrifuged at 3000 rpm for 5 min). A flame ionization detector (FID) and a column capillary Agilent J&W DB-WAX UI, 30 m × 0.25 mm, 0.25 μm (p/n 122-7032UI) was used. The GC operated using a nitrogen gas at 1 mL/min, while the combustion hydrogen, and gas and air mixture were adjusted to 40 mL/min and 400 mL/min, respectively. The GC operated at a temperature program of 60 °C (1 min), a ramp rate of 12 °C per hold time, and up to 220 °C (3 min), and a final FID detector temperature of 250 °C. The data produced was collected and processed using ChemStation CDS software. Calibration curves were produced for all solvents investigated in this study. The calibration equations derived from this exercise were used to calculate the concentration levels of the compounds in the fermenting liquor at 24-h intervals for the duration of the fermentation process.

3. Results

3.1. Phylogenetic Analysis

A total of 554 bp from CL11A and CL11D genomic extracts were obtained from pure cultures of bacteria isolated from the soil surrounding the roots of beans and ZM 3A (600 bp) from fermented sugarcane juice were submitted to NCBI BLAST. These samples were subjected to an identification process, which showed they were most similar to *Leuconostoc mesenteroides* and *Weissella cibari*: 100% for CL11A and CL11D, respectively. The ZM 3A was identified as *Lactobacillus plantarum*. Evolutionary analysis was conducted using MEGA X software and the phylogenetic relationships were analyzed using the neighbor-joining method. The analysis showed that all three identified bacteria shared a common ancestry as they are members of the *Lactobacillales* class. The isolates from the roots of beans both belong to the genus *Leuconostocacea*, whilst isolate ZM 3A belongs to the genus *Lactobacillaceae*. The phylogenetic relationships are shown in Figure 1.
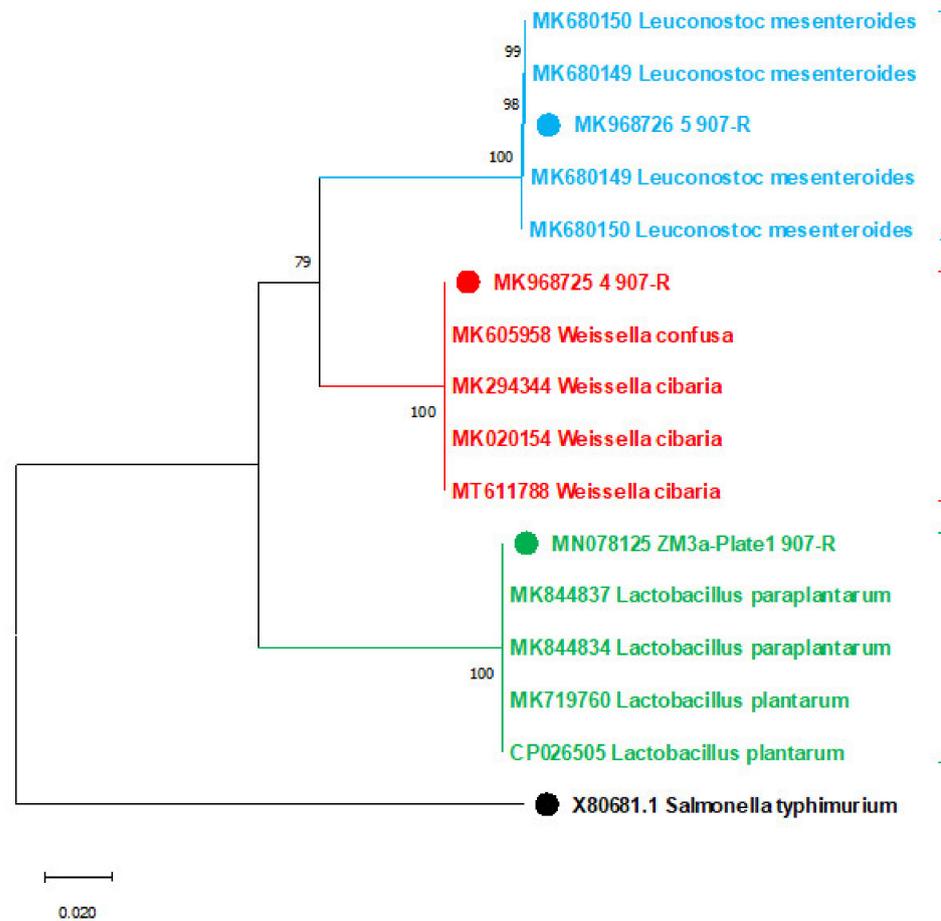
Figure 1. Phylogenetic tree of bacterial isolates from roots of beans and sugarcane juice.

3.2. Ethanol Production by Lactobacillus plantarum

The fermentation performance of *L. plantarum* is shown in Figure 2. Several bacterial isolates from sugar cane juice were observed, but the selected *L. plantarum* (ZM3A) demonstrated the highest ethanol production (39.7 g/L). However, this bacterium showed poor sugar utilization compared to the ABE isolates (shown in Figures 3a and 4a), with 13.1 g/L remaining after 12 days of incubation. In contrast, the ABE isolates reduced the sugar concentration average to below 7.33 g/L (see Figures 3a and 4a).

The final pH levels decreased from 6 to as low as 3.31 in the fermentation vessel, while that of ABE fermentation decreased and stabilized at pH 3.67. Microbial growth was also lower compared to the average for ABE-producing isolates (see Figures 3b and 4b), with the final cell density in each case being 1.9×10^{10} CFU/mL versus 2.12×10^{12} CFU/mL, respectively. The observed product assay was better for ethanol production, with a record high *L. plantarum* production of 39.7 g/L (see Figure 2), while the highest concentration for ABE isolates was 7.26 g/L (See Figures 3a and 4a.)
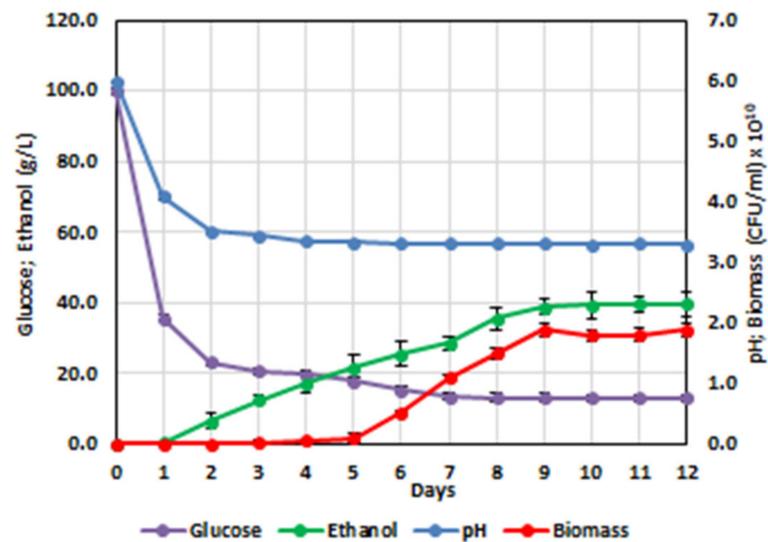
Figure 2. Fermentation performance of ethanol-producing *L. plantarum*.

3.3. Comparison of ABE Production for Leuconostoc mesenteroides and Weissella Cibari

The sugar utilization and reduction from the initial 100 g/L was observed to be almost equal for both isolates at the end of the fermentation period: the residual sugar for *L. mesenteroides* was 7.51 g/L, and for *W. cibari* it was 7.14 g/L. Total solvents (ABE) produced after the full incubation period was 7.31g/L for *L. mesenteroides* and 7.21 g/L for *W. cibari*. Ethanol production was dominant in both fermentation processes, but it was more pronounced in *W. cibari*; however, the acetone and butanol were significantly lower in comparison. Total acids (acetic and butyric) produced during reaction were high (0.63 g/L) with *L. mesenteroides* compared to the 0.53 g/L produced by *W. cibari*. Acetic acid increased to a peak, then reduced towards the end of the incubation period in both fermentation processes, although this trend was more consistent in *L. mesenteroides* than *W. cibari*. As shown in Figures 3 and 4, microbial growth and pH followed a similar trend in both fermentations, but the growth rate for *W. cibari* was slightly lower than that of *L. mesenteroides*.
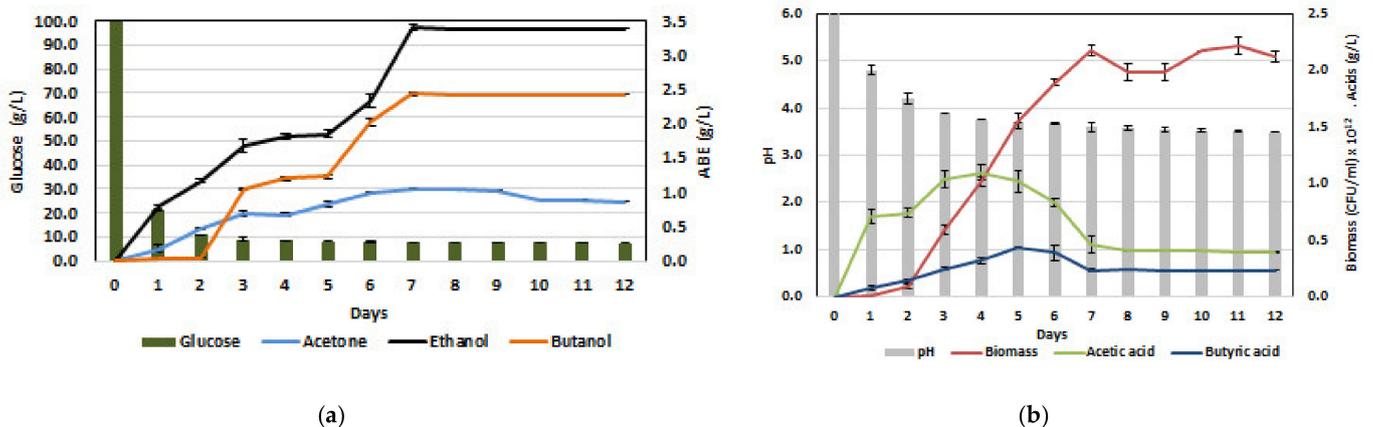

(**a**)

(**b**)

Figure 3. Fermentation results from isolate *L. mesenteroides*. (**a**) Sugar consumption and ABE concentration during incubation. (**b**) pH, microbial growth and acids production during incubation.
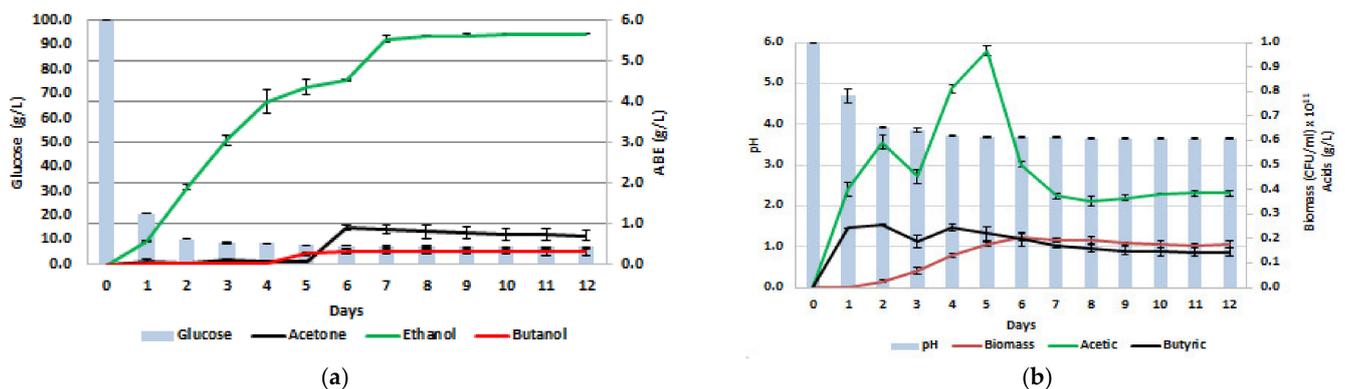
Figure 4. Fermentation results from isolate *W. cibari*. (**a**) Sugar consumption and ABE concentration during incubation. (**b**) pH, microbial growth and acids production during incubation.

3.4. Co-Culture Fermentation Using Lactobacillus plantarum and Leuconostoc mesenteroides

The higher ABE production and substrate conversion rates of *L. mesenteroides* resulted in its selection for co-culture fermentation with *L. plantarum*. A comparison of the sugar utilization across all three monoculture fermentations showed that the lowest conversion rates were in the co-culture fermentation. As shown in Figure 5a, the sugar levels in the reactor reduced from 100 g/L to almost 20 g/L over more than 3 days, yet the same level of sugar reduction was achieved in one day in the case of *W. cibari* and *L. mesenteroides* (see Figures 3a and 4a) The same sugar utilization took exactly 3 days in the case of *L. plantarum* ethanolic fermentation, which was a slightly shorter period than in the co-culturing fermentation.

Acetone levels fluctuated throughout the fermentation period. The production of ethanol was highest in co-culture fermentation, with a final concentration of 44.74 g/L, while that of the monocultures was 39.7 g/L (*L. plantarum)* and 5.7 g/L (*L. mesenteroides*). However, butanol concentrations were reduced in the co-culture fermentation of the two strains of bacteria when compared to monocultures of ABE-producing strains. Butyric and acetic acid concentrations also fluctuated throughout the fermentation period, and corresponded to butanol and ethanol production patterns. However, acetic acid levels were relatively higher than butyric acid levels.

There was an obvious bias towards ethanol production in the co-culture at the expense of acetone and butanol. The pH patterns in the co-culture were similar to trends observed in monocultures of ABE fermentation. The final pH in the co-culture fermentation was 3.62. With the monoculture cases for *L. plantarum* it was 3.3, while that of the ABE isolates averaged 3.56. Despite a similarity in the microbial growth and pH patterns of monocultures of ABE isolate *L. mesenteroides* and co-culture fermentations, the product formation patterns were biased towards ethanol production and similar to monoculture fermentation of the ethanol-producing *L. plantarum*. The product formation trends indicate the likely dominance of the *L. plantarum* enzymatic activities in the co-culture fermentation although an explanation for the microbial growth and pH change patterns could not be immediately determined.

Microbial growth patterns in co-culturing closely resembled that of the ABE-producing strain *L. mesenteroides*, but deviated more than the ethanol-producing strain *L. plantarum*. A similar pattern was observed with pH changes, where the co-culture pattern resembled the ABE strain more than the ethanol strain. However, the product yields were more biased towards ethanol production than the ABE solvents in the co-culture reactors.
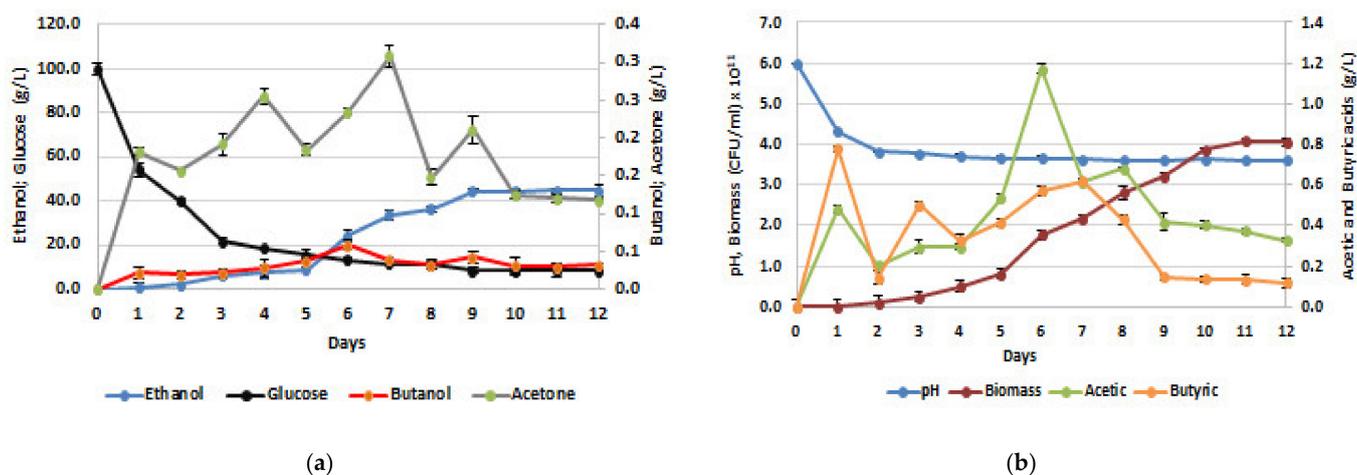
(**a**)   (**b**)

Figure 5. Coculture fermentation performance of *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. (**a**) Glucose consumption and final products (solvent) concentrations. (**b**) Acid production, pH and microbial growth trends.

In summary, Table 1 shows a comparative analysis of the yield of the ABE solvents observed in this study compared with previous studies that had produced the three solvents.

Table 1. Comparative analysis of ABE solvents (product yield) observed in this study versus previous similar studies.

Microorganism	Substrate	Product	* Yield (g/g)	Reference
Lactobacillus plantarum	Glucose	Ethanol	0.40 (0.04)	This study
Weissella cibari	Glucose	Ethanol	0.06 (0.00)	This study
Leuconostoc mesenteroides	Glucose	Ethanol	0.03 (0.00)	This study
Lactobacillus plantarum + *Leuconostoc mesenteroides*	Glucose	Ethanol	0.45 (0.03)	This study
Sacharomyces cerevisiae	Sugar beet molasses	Ethanol	0.40	[30]
Recombinant *Sacharomyces cerevisiae* 1400 (pLNH33)	Glucose + Xylose	Ethanol	0.46	[31]
Thermoanaerobacterium AK17	Cellulose	Ethanol	0.40	[32]
Weissella cibari	Glucose	Butanol	0.003 (0.00)	This study
Leuconostoc mesenteroides	Glucose	Butanol	0.02 (0.00)	This study
Lactobacillus plantarum + *Leuconostoc mesenteroides*	Glucose	Butanol	0.0003 (0.00)	This study
Engineered *Clostridium tyrobutyricum*	Sucrose	Butanol	0.06	[33]
Weissella cibari	Glucose	Acetone	0.01 (0.001)	This study
Leuconostoc mesenteroides	Glucose	Acetone	0.01 (0.00)	This study
Lactobacillus plantarum + *Leuconostoc mesenteroides*	Glucose	Acetone	0.001 (0.00)	This study
Clostridium acetobutylicum JB200	Sucrose	Acetone	0.14	[34]

This study's results are reported as means ± (standard deviation), *n* = 3. * Yield reported as amount of product per amount of hexose sugars added to the reactor.

4. Discussion

This study isolated and screened organisms present in the soils around the roots of beans and in sugarcane juice. The selective screening utilized alkalized sodium nitroprusside to test for the presence of ketone and acetone. The red-color complex that is visible when pure cultures were introduced into the compound are the consequence of the reaction of nitroprusside with the free –SH groups in the amino acids and/or from the cysteine amino acids in the available proteins present in the cells. Notably, several studies have indicated the bactericidal effect of sodium nitroprusside [35–38]. This bactericidal effect of sodium nitroprusside is suggested to be the result of the release of its highly reac-

tive nitric oxide (NO) [39]. The oxidation or reduction of NO produces reactive nitrogen species (RNS) compounds that are known to react with some components of the cells including thiols, lipids and metals, which results in metabolic inhibition, membrane and DNA damage [40]. Thus, sodium nitroprusside is considered to be an effective screening agent in this bioprospecting study and the likely reason for the identification of only the most robust ABE-producing bacteria in this environment, thereby drastically reducing the number of potential isolates. Moreover, the acetone test is a good indicator for the presence of both acetone and butanol-producing bacteria, given that the anaerobic shunt of the glycolytic pathway of starch degradation produces the intermediate acetyl CoA, which is invariably broken down to acetone, butanol and butyrate, depending on the prevailing conditions [41].

Leuconostoc mesenteroides and *Weissella cibaria* are heterolactic fermentative bacteria and are capable of fermenting glucose and various hexose sugars by utilizing either the hexose monophosphate and pentose phosphate pathways [42,43] as well as other metabolic pathways such as the conversion of citrate to diacetyl and acetoin [44]. Acetoin is a methyl ketone, that is, butan-2-one substituted by a hydroxyl group at the chemical structural position "3". It is a metabolite within the biochemical pathways leading to the formation of acetone and butanol. *L. mesenteroides* has been previously indicated in the fermentation and souring of vegetables including cucumbers and cabbage, producing pickles and sauerkraut, respectively [45]. The bacteria *L. mesenteroides* play an important role in several industrial and food fermentations and are widespread in the natural environment; therefore, their presence in this environment surrounding the root of beans is not surprising. However, studies linking *L. mesenteroides* directly to acetone and butanol production appear to be scarce and further investigation may be required. *Weisella* spp. was previously considered a species of the *Leuconostoc paramesenteroides* group [46]. In a taxonomic study done by Bjorkroth et al. [47], it was shown to be the dominant species in fermented food such as kimchi. More recently, there has been a focus on their probiotic potential [48,49]. *Weissella cibaria* has also been isolated from several environments including the Thai fermented fish product, plaa-som [50] and a Chinese liquor, Xiaoqu [51]. Previous studies have indicated that the strain *Weissella confusa* BR0216-18 is capable of tolerating 3–4% butanol after long-term adaptation [52].

With the sugarcane juice, bacteria were similarly screen with sodium nitroprusside and Schiff's reagent and only one bacteria (ZM 3A) tested positive for high levels of acetaldehyde, the alternative shunt metabolite in the anaerobic progression of the glycolytic pathway of glucose degradation. Excess acetaldehyde is converted to ethanol during fermentation. The bacteria, ZM3A was identified as having 100% similarity to *Lactobacillus plantarum* strain DHYY14. *L. plantarum* is considered a versatile lactic acid bacterium that has been isolated previously from diverse niche environments including from fish, meat, and several fermented products. It has also found application in controlled fermentations where specific products and properties are desired [53,54]. Although, *L. plantarum* has been previously described as having probiotic properties [55–57], there does not seem to be previous links between this bacterium and ABE fermentation. However, one previous study attempting to isolate *Clostridium acetobutylicum*, eventually identified strains derived from screening as lactic acid bacteria (LAB), which demonstrated tolerance and the ability to grow in butanol concentrations of 2–5% (*v/v*), indicating the potential of many strains of LAB towards butanol tolerance [58]. This is similar to the work presented in this study. The goal was to isolate species of *Clostridium* and instead LAB were isolated. More recently, Russmayer et al. described a two-step production of 2-butanol by *L. dioliworans* [59]. Their study highlighted the inability of *L. dioliworans* to produce meso-2,3-butanediol and the corrective measures employed to overcome this challenge, by employing a two-step cultivation process with *Serratia marcescens* providing meso-2,3-butanediol. Overall, the process yielded 10 g/L of butanol. However, their work was also significant in that the yield was improved by 34% through the genetic engineering of *L. dioliworans* to overexpress

the endogenous alcohol dehydrogenase pduQ. This study and the present one, support the potential of utilizing LAB in ABE fermentation with further progressive investigations.

This present study monitored growth, substrate utilization (through glucose depletion), pH changes as well as the metabolite production of acetic acid, butyric acid, acetone, butanol and ethanol during fermentation. Lag phases at the beginning of fermentation, tended to last for 24 h suggesting a period of acclimation and the synthesis of enzymes for biomass growth and an increase as well as involvement in fermentation [60]. The increased biomass and its stability throughout fermentation suggest a tolerance to pH changes and increased alcohol concentrations. Previous studies have shown *Lactobacillus plantarum* pH tolerance to range between 3.2–4.0 and concentrations of ethanol as high as 13% (*v/v*) [61]. Other authors have observed demonstrated tolerance to low pH of strains of the same species as those used in this study [62,63]. Thus, in this batch experiment the ability of the bacteria to grow at pH 3.3 is an indication of its high-acid tolerance, a quality of significance in bio-engineering and fermentative bio-processes [61,64].

Although monocultures of bacteria have often been employed in fermentation processes, many biological processes benefit from the presence of more than one species of bacteria in a system [65], especially since normal microbial habitats include several species of microorganisms that contribute enzymes to different biochemical pathways towards substrate utilization. Luo et al. explains that the most significant advantage of a co-culture system is that the combined metabolic capacities of the two microorganisms enhance substrate utilization and the production of specific products [66]. Remarkably, the co-culture fermentation demonstrated lower sugar utilization rates compared with monocultures; this trend will require further investigation. A bias towards ethanol production at the expense of acetone and butanol, was observed in the co-cultures of ABE-producing *L. mesenteroides* and ethanol-producing bacteria *Lactobacillus plantarum* (see Table 1). This was evidenced through the increased levels of acetic acid. This observation bears a similarity to observations made by Liu et al. while investigating the effect of acetic acid on ethanol production using two different mutant strains of *Zymomonas mobilis,* for which the results indicated that acetate-tolerant strains produced higher levels of ethanol [67].

In this study, the superior production of ethanol is a result of the adaptation of *Lactobacillus plantarum* to low pH levels caused by acetic acid.

5. Conclusions

Research efforts towards the identification of superior bacterial strains that can be employed in the production of clean and renewable biofuels have steadily increased, and such research is facilitated by bioprospecting niche environments. This study has demonstrated that it is possible to isolate other bacteria species that demonstrate tolerance to low pH and a high concentration of metabolites including ethanol by identifying three uncommon bacteria strains for the production of ABE and ethanol. They are strains of *Leuconostoc mesenteroides* (CL 11A), *Weissella cibaria* (CL 11D) and *Lactobacillus plantarum* (ZM 3A). Moreover, it was also observed that there was a bias towards ethanol production in co-cultures of ethanol-producing *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. This will require further investigation to expand our understanding of the potential value of lactic acid bacteria for biofuel production. Future research may focus also on attempting to eliminate this bias by using other sugars as substrates.

However, this study found that *Lactobacillus plantarum* (ZM 3A) shows great promise as an industrial strain, demonstrating superior ethanol production and strong adaptation to acidic environments.

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