Solid-state Fermentation of Cassava Roots using Cellulolytic-type Alkaliphilic *Bacillus* spp. Cultures to modify the Cell Walls and assist Starch release

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Abbreviations:

Cell wall material (CWM); Endogenous microflora (EM); Maximum Recovery Diluent (MRD); Wide-angle x-ray scattering (WAXS) spectroscopy

Abstract

To improve cassava starch extraction by wet milling, solid-state fermentation of ground roots using cellulolytic-type alkaliphilic Bacilli spp., *Bacillus akibai, B. cellulosilyticus* and *B. hemicellulosilyticus* was investigated. Enzyme assay and scanning electron microscopy indicated that *Bacillus* spp. production of extracellular cellulase and polygalacturonase caused formation of micropores through the root parenchyma cell walls and exposed the embedded cellulosic network. Gas chromatography data of the cell wall constituent sugars remaining after fermentation and Fourier transform infrared data indicated that the *Bacillus* treatments reduced the levels of pectin and, hemicellulose and to lesser extent cellulose. Wide angle X-ray scattering data indicated that the *Bacillus* spp cell wall degrading enzymes had partially hydrolysed the amorphous fractions of the cell wall polysaccharides. All the *Bacillus* spp. treatments improved starch extraction by 17-23% compared to fermentation with endogenous microflora. *B. cellulosilyticus* was most effective in disintegration of large root particles and as result released marginally the most starch, probably due to it having the highest cellulase activity. Solid-state fermentation using cellulolytic-type *Bacillus* spp. is therefore a promising to technology to improve the efficiency of cassava wet milling cell wall disintegration and consequent starch yield without use of commercial cell wall degrading enzymes or polluting chemicals.

Keywords Alkaliphiles; *Bacillus;* Cassava; Cell wall polysaccharides; Solid-state fermentation

Introduction

Cassava (*Manihot esculenta* Crantz L.) is a starchy root crop cultivated worldwide for direct human and livestock consumption and has potential as feedstock for biofuels and other bio-based materials [1]. Cassava starch is also one of the major starches, contributing 12% of the global starch production in 2011, second only to maize [2].

Isolation of starch from cassava roots invariably involves milling of the roots to liberate starch granules from within the parenchyma cells. Industrially, wet milling of the fresh roots is preferred over dry milling due to its lower energy demand [3]. The liberation of starch granules from within the root parenchyma cells by wet milling is invariably inefficient, resulting in some 15-20% of the original root weight as solid waste containing approx. 50% starch [4]. Thus, improving starch extraction yield is of critical importance.

Wet milling of cassava both at industrial and domestic scale is normally combined with fermentation. Fermentation of cassava roots aids reduction of postharvest losses, improves starch extraction through the action of extracellular cell wall degrading enzymes and facilitates degradation of toxic cyanogenic glycosides [5]. With specific regard to cell wall degradation, it has been found that with endogenous microorganism-type fermentation, cassava root softening is mainly due to degradation of the cell wall pectin through the action of pectin-methylesterase and pectate-lyase [6]. Also, treatment of milled cassava roots with commercial cellulolytic and hemicellulolytic enzymes prior to endogenous lactic acid bacteria fermentation by organisms including *Lactobacillus plantarum* was shown to exert a complementary degradatory action on the cell walls [7]. Fermentation with starter cultures has been less studied. *Saccharomyces cerevisiae* strain 2Y48P22 in submerged fermentation either singly or in combination with *Bacillus cereus,* which is predominant in a particular traditional cassava fermentation, was found to result in the softest cassava roots [8]. This *S. cerevisiae* strain exhibited pectinase activity in a model system.

Alkaliphilic *Bacillus* spp. produce several alkaline-stable extracellular cell wall degrading enzymes of industrial importance, including cellulases, xylanases and pectinases [9, 10]. Due to the stability of these enzymes under conditions of high salinity, pH and ionic strength, biocatalysis using alkaliphiles and their enzymes is of significant industrial interest [11]. The application of alkaliphilic *Bacillus* spp. solid-state fermentation to cassava roots has not been investigated. The aim of this study was to establish whether fermentation by alkaliphilic *Bacillus* spp. producing cell wall degrading enzymes could weaken the cassava parenchyma cell walls and as a consequence result in more effective cell disintegration during wet milling.

Materials and methods

Materials

Cassava roots of sweet variety *South Africa* were harvested from eleven months old plants grown in a single field at Tonga, Mpumalanga Province, South Africa. Three different alkaliphilic *Bacillus* species were investigated. Selection of alkaliphilic bacteria was based on their ability to produce extracellular alkaline-stable cell wall degrading enzymes with no or minimal amylase activity [10]. *Bacillus akibai* (ATCC 43226) was from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). *Bacillus cellulosilyticus* (JCM 9156) and *Bacillus hemicellulosilyticus* (JCM 9152) were from the Japan Collection of Microorganisms (JCM) (Koyadai, Japan).

Preparation of Alkaliphilic *Bacillus* spp. Inocula and Culture conditions

B. akibai was cultured on ATCC 1513 PY-CMC medium (carboxymethyl cellulose (10 g), Becton and Dickinson 211910 polypeptone peptone (5 g), yeast extract (5 g), NaCl (5 g), KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (2 g), agar (15 g), distilled water 1 L, and 100 ml 10% (w/v)

Na_2CO_3 soln. was added aseptically after autoclaving). It was incubated aerobically at 30°C for 18 h. *B. cellulosilyticus* and *B. hemicellulosilyticus* were cultured on Horikoshi I agar (glucose (10 g), BD 211910 polypeptone peptone (5 g), yeast extract (5 g), K_2HPO_4 (1 g), $MgSO_4.7H_2O$ (0.2 g), agar (15 g), distilled water 900 mL, and 100 mL 10% (w/v) Na_2CO_3 soln. was added aseptically after autoclaving). It was incubated aerobically at 37°C for 18 h. Harvested *Bacillus* spp. cells were suspended in 8 mL sterile Maximum Recovery Diluent (MRD) (0.1% peptone and 0.85% NaCl) and centrifuged at 2,400 g for 20 min at 4°C. Concentrated *Bacillus* spp. cells were standardised to 9.0 McFarland standard using a DEN-1 McFarland densitometer (Grant Instruments, Cambridge, UK). To prepare the *Bacillus* spp. in combination inoculum, equal portions of standardised single spp. inocula were combined. Horikoshi I agar was used for the *Bacillus* spp. in combination inoculum.

Alkaliphilic *Bacillus* spp. Solid-state Fermentation of Cassava

The cassava roots were washed, peeled, chopped into chips, then stored frozen as described [12]. When required, they were thawed and ground using an electric rotary meat mincer fitted with an 8 mm opening plate. The ground cassava (200 g) was mixed thoroughly with 40 mL sterile 10% (w/v) Na_2CO_3 soln. in 1 L plastic buckets. Inclusion of the Na_2CO_3 raised the cassava surface pH to pH 10.5. Na_2CO_3 was used for this purpose as it was found to enable high xylanase production with *Bacillus* solid-state fermentation using wheat bran as substrate [13]. Standardised 8 mL inocula of *B. akibai*, *B. cellulosilyticus*, *B. hemicellulosilyticus* and *Bacillus* spp. in combination were added. To the control which as not inoculated with *Bacillus* spp., 8 mL sterile MRD was added. This treatment was chosen as the control (hereafter referred to as the endogenous microflora (EM) fermentation) because when cassava roots are processed by wet milling they invariably undergo a spontaneous fermentation, commonly referred to as retting [6]. Furthermore, *Bacillus* spp. with various

extracellular enzymic activities have been isolated from this type of cassava fermentation [14], so the requirement was to distinguish between the effects of inoculation with the alkaliphilic *Bacilli* and the EM microflora.

The buckets were covered with aluminium foil and incubated at 30^{o}C for 72 h. Fermentation conditions (pH, temperature and Na^{+} concentration) were selected on the basis of the recommended conditions for optimal growth of alkaliphilic *Bacillus* spp.by ATCC (www.lgcstandards-atcc.org/products) and JCM (www.jcm.riken.jp/cgi-bin/jcm/jcm) and on conditions for extracellular xylanase production by solid-fermentation of alkaliphilic *Bacillus* sp. on wheat bran [13,15]. The fermented cassava pieces were then pulverised in a Waring blender for 1 min using low and high speed, each for 30 s. The pulp was suspended in 5x its volume distilled water and wet milled into a slurry using a Retsch EZ200 wet mill (Haan, Germany) fitted with a 2000 µm mesh screen at 12,000 rpm.

Preparation of Starch-rich Fraction, Fibre-rich Fraction and Cell Wall Material (CWM)

The fermented slurry was separated by sieving into a fibre-rich fraction, which remained on a 106 µm mesh sieve and a starch-rich fraction, the filtrate. After washing several times, the fibre-rich and starch-rich fractions were dried in a forced draught oven and milled [12].

To prepare CWM from the fermented cassava, starch remaining in the fibre-rich fractions was removed by hydrolysis using thermostable α-amylase, as described [16]. The CWM was freeze dried and then milled as described [12] and then characterised.

Analyses

Determination of Endogenous Microflora and Alkaliphilic *Bacillus* spp. growth on Cassava

For alkaliphilic bacteria enumeration, ground cassava pieces were alkaline treated, inoculated (except the EM control treatment) and incubated as described under 2.3. At 0 h, and after every 12 h, triplicate samples (25 g) of each treatment were suspended in 225 mL MRD and homogenized using a Stomacher for 30 s. Bacteria in the fermentations were enumerated using recommended procedures of ATCC (www.lgcstandards-atcc.org/products) and JCM (www.jcm.riken.jp/cgi-bin/jcm/jcm). Diluted samples were cultured on the following growth media: *B. akibai* on ATCC 1513 PY-CMC agar at 30°C for 48 h; *B. cellulosilyticus* and *B. hemicellulosilyticus* on Horikoshi I agar at 37°C for 48 h; *Bacillus* spp. in combination and EM on Horikoshi I agar at 30°C for 48 h. Distinct morphological differences between the endogenous bacteria and alkaliphilic *Bacillus* spp. colonies (which were whitish, translucent, with large flat surface and irregular margins) aided enumeration of the presumptive alkaliphilic *Bacillus* spp. counts.

pH

The pH of the fermenting cassava was determined after diluting 1:2 with distilled water and thoroughly mixing.

Determination of Beta-1,4-endoglucanase and Endo-polygalacturonase activities of the Cultures

Ground cassava pieces were alkaline treated, inoculated and incubated as described under 2.3. At 24 h intervals, samples (50 g) of each treatment were removed and centrifuged at 10,000 g for 15 min at 4^{o}C. The cell-free supernatants (crude enzyme extracts) were

immediately assayed, according to [17] with modifications. Alkaline β-1,4-endoglucanase and endo-polygalacturonase activities were assayed using 1% (w/v) carboxylmethylcellulose-methyl salt (high viscosity) (BDH, Poole, UK) and 5% (w/v) pectin (type AS 509, Tate & Lyle, London), respectively. The substrates were suspended in 0.1 M glycine-NaOH buffer, pH 10.5. Substrate solutions (20 mL) were reacted with 0.2 mL crude enzyme extracts for 10 min at 37^{o}C and 160 s^{-1} shear rate in an Anton Paar Physica MCR 101 rheometer (Graz, Austria). For controls (enzyme blanks), 0.2 mL sterile 10% (w/v) Na_2CO_3 solution was added. Beta-1,4-endoglucanase and endo-polygalacturonase activities were recorded as percentage viscosity reduction (% η-reduction). These activities were quantified in terms of the activities of Viscozyme L and Pectinex Ultra SP-L, respectively (kindly provided by Novozymes SA, Johannesburg, South Africa). According to the Novozymes data sheets, Pectinex Ultra SP-L activity is mainly polygalacturonase and Viscozyme L is primarily endo-β-glucanase (www.gusmerenterprises.com). Viscozyme L was reacted with 1% (w/v) carboxylmethylcellulose and Pectinex Ultra SP-L with 5% (w/v) pectin under the same assay conditions.

Starch content of the Starch-rich and Fibre-rich Factions

These were determined using the Megazyme International Total Starch Assay kit (available at www.megazyme.com).

Fibre-rich Fraction Particle Size

This was determined by sieving 5 g wet fibre-rich fraction through 250 µm, 106 µm and 25 µm mesh sieve sizes using 2 L distilled water. The particles retained on the sieves were dried according to the AACC Moisture-Air-Oven Method 44-15A [18].

Gas chromatography (GC) characterisation of CWM

GC analysis of the neutral sugar composition of the destarched CWMs and spectrophotometric analysis of their uronic acid content were performed by Englyst Carbohydrates Ltd (Southampton, UK), as described [12].

Scanning Electron Microscopy (SEM) of CWM

CWM was neutralised by suspending in 300x its volume of distilled water, collected over a 106 µm mesh sieve and freeze dried. The dry residue was mounted on an aluminium stub, carbon coated (<0.5 nm thickness) and viewed using a JEOL JEM-8700 SEM (Tokyo, Japan). For each replicate sample, five specimens were randomly collected and eight locations per specimen viewed.

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) and Wide-angle X-ray scattering (WAXS) Spectroscopy of the CWM

For ATR-FTIR and WAXS measurements, the milled dry CWMs were equilibrated for 7 days at approx. 25°C in a desiccator containing silica gel. Diamond crystal ATR-FTIR was performed on this CWM material, as described [12].

WAXS measurements on the CWM were performed as described [12]. Material relative crystallinity was estimated as the ratio of the integrated area of crystalline peaks to the total integrated area above a straight baseline, as described [19].

Statistical Analyses

All experiments were replicated at least twice. Microbiological analyses and extracellular enzyme assays were replicated at least three times. Data were analysed using

one-way analysis of variance (ANOVA). Fisher's Least Significant Difference (LSD) test was used to determine significant differences between means.

3. Results and Discussion

Growth of Alkaliphilic *Bacillus* spp. and EM on Cassava Pieces

There was rapid growth of the alkaliphilic *Bacillus* spp. over the first 36 h of solid-state fermentation on the alkaline cassava pieces (Fig. 1A). By 48 h, the *B. akibai, B. cellulosilyticus, B. hemicellulosilyticus* and *Bacillus* spp. in combination counts had all reached their maxima of 11.3, 11.0, 10.9, and 10.6 $\log_{10}$ colony forming units (cfu)/g, respectively. Hence, cassava roots alone was thus a suitable substrate for growth of the *Bacillus* spp. This finding is consistent with research that showed wheat bran was a good substrate for xylanase production by *Bacillus pumilus* [15]. This was attributed to the presence of the nutrients protein, amino acids and minerals in the wheat bran, all which are present in cassava roots (USDA). Moreover, other research obtained high xylanase production by solid-state fermentation of alkaliphilic *Bacillus* sp. AR-009 on wheat bran just with added K, Mg and Ca ions, at concentrations of 22, 2 and 3 mg/100 g, respectively [13]. These minerals are present in cassava roots at several times these concentrations, approx. 271, 21 and 16 mg/100 g, respectively (USDA FoodData Central www.fdc.nal.usda.gov).

Concerning the EM (control) treatment, despite its low count at the start of fermentation (4.4 $\log_{10}$ cfu/g) there was, surprisingly, also considerable microbial growth over the first 36 h of fermentation with the result that the cell count at 48 h of 10.8 $\log_{10}$ cfu/g was in the same range of the *Bacillus* spp. fermentations (Fig. 1A). As the Na_2CO_3 treated cassava pieces had a surface pH of 10.5 but were not sterile, microbial colonies isolated on Horikoshi agar from the EM treatment were considered presumptive lactic acid bacteria due to the strong decline to pH 5.5 (Fig. 1B). Only alkaliphilic microorganisms possess the ability

to grow well on Horikoshi agar [20]. However, lactic acid bacteria are known to possess the ability to survive alkaline pH [21].

Specifically concerning changes in pH during incubation, with the *Bacillus* spp. inocula, the pH change during incubation followed a similar pattern, i.e. an initial decrease followed by a slight increase and then generally a slight decrease (Fig. 1B). Importantly, throughout the fermentations using the alkaliphilic *Bacillus* spp. the pH did not drop below pH 8.0, indicating that the alkaliphilic bacteria predominated in these fermentations. In contrast, during the EM fermentation, the pH dropped below 7.0 after only 24 h of incubation. This was presumably as a result of acid production by the endogenous bacteria.

Extracellular Enzyme Activities of the *Bacillus* spp. and EM during Fermentation

By 48 h incubation, fermentation of cassava with any of the *Bacillus* spp.+EM, either singly or in combination, resulted in substantially ($p < 0.05$) higher cellulase activity compared to the EM fermentation (Fig. 2A1). At 72 h, *B. cellulosilyticus*+EM produced by far the highest level of cellulase, 628 ppm (Viscozyme equiv.), some 4.5 times that by *B. akibai*+EM, the next highest producer (Fig. 2A2). These findings are in agreement with *B. cellulosilyticus* being a cellulase producer and *B. akibai* being a CMC-ase producer [10].

Concerning endo-polygacturonase activity, by 48 h fermentation *B. akibai*+EM fermentation had slightly but significantly ($p < 0.05$) more endo-polygalacturonase activity compared to any of the other *Bacillus* spp.+EM fermentations either singly or combination (Fig. 2B1). Polygalacturonase activity has been reported in some *Bacillus* spp., for example *B. sphaericus* [22]. Also, other work showed that some *Bacillus* isolates from cassava dough fermentation exhibited polygalacturonase and pectin esterase activity [14]. Polygalacturonase production was not detectable in the EM fermentation. Notably, with the *B. cellulosilyticus*+EM and *B. hemicellulosilyticus*+EM fermentations, polygalacturonase

activity was only clearly detectable after 72 h incubation (Fig. 2B2). This is probably because *B. cellulosilyticus* does not possess pectate lyase (PL) or glycosyl hydrolase family 28 (GH28) member genes, which is indicative of the microorganism's inability to produce extracellular pectinases [23].

Effect of Alkaliphilic *Bacillus* spp. Fermentation on Cassava Cell Walls

With all the *Bacillus* spp.+EM fermentations excepting the *B. akiba*+EM fermentation, there was a substantial reduction ($p<0.05$) in large cassava particles (>250 μm) in the fibre-rich faction and a concomitant increase in the smaller particle size fractions when compared to the EM fermentation (Table 1A). It has been similarly found that the action of the commercial cellulolytic enzymes combination with lactic acid fermentation resulted in a reduction in cassava CWM particle size [7]. All the *Bacillus* spp.+EM fermentations also resulted in substantially less ($p<0.05$) CWM remaining (1.4-1.7 g/100 g cassava root) compared to the EM fermentation (2.7 g/100 g). These data indicate that some of the cassava cell wall polymers had been hydrolysed by the extracellular cellulase and polygalacturonase enzymes of the *Bacillus* spp.

The neutral sugar composition of the CWMs that remained after the *Bacillus* spp.+EM and EM fermentations comprised glucose (average 62%), xylose (average 14%), galactose (average 6%), arabinose, mannose, fucose and rhamnose in decreasing order (Table 1B). This order differs somewhat from the reported original sugar composition of cassava cells, which comprised glucose (64%), mannose (8%), xylose (6%), galactose (5%), arabinose, fucose and rhamnose in descending order [24]. It is significant that glucose, xylose and galactose were the major neutral sugars remaining in both the EM and *Bacillus* spp. fermented cassava CWMs. This indicates that cellulose and probably xyloglucans were the

major non-starch polysaccharides (NSPs) remaining after enzymic hydrolysis. With all the fermentation treatments, total NSP sugar recovery was low, approx. 38-46% but reasonably consistent. The low recoveries can be attributed to the NSPs remaining after hydrolysis being bound to cell wall lignin [7], which probably prevented their complete hydrolysis during assay. Lignin is primarily from the cassava root xylem and central vascular fibre tissue cell walls [25].

Generally, fermentation of cassava with all the *Bacillus* spp. substantially reduced ($p<0.05$) the levels of NSP component sugars in comparison with the EM fermentation (Table 1B). Overall, for the *Bacillus* spp. fermentations the percentage reduction in arabinose content was the largest, followed by galactose. This indicates that NSPs rich in these sugars were degraded to the greatest extent by extracellular hydrolytic enzymes produced by the *Bacillus* spp. In cassava cell walls, the pectin is composed mainly of galacturonic acid and galactose residues, while the hemicellulose fraction comprises mainly xylose, arabinose, and galactose [13]. Therefore, cassava fermentation using alkaliphilic *Bacillus* spp. appears to have primarily resulted in the loss of the cell wall pectin and hemicellulose components, with the cellulose being hydrolysed to a proportionally lesser extent (Table 1B). Hydrolysis of pectin is consistent with the polygalacturonase activity exhibited by all the *Bacillus* spp. (Fig. 2B). Unfortunately, the xylanase-type activity of the Bacillus spp. was not measured. However, as mentioned, *Bacillus* spp. can have high xylanase activity in solid-state fermentations [13,15]. Quantitatively, by far the greatest reduction in residual sugar levels was with glucose, 3-4 times higher than any of the other sugars (Table 1B). This indicates that cellulose was also degraded. This is consistent with the cellulase activity exhibited by all the *Bacillus* spp. (Fig. 2A).

SEM revealed that all the *Bacillus spp.*+EM fermentations, especially the fermentations involving *B. cellulosilyticus,* and *B. hemicellulosilyticus* (Fig. 3C-E), resulted

in the exposure of the cell wall fibres (CWF) and cell wall fibre rupturing (RCM). These features were absent with the EM fermentation (Fig. 3A). Lignocellulosic cell wall deconstruction (breakdown) as a result of exposing the embedded crystalline cellulose has been associated with a reduction in cellulose relative crystallinity [26]. There was evidence that some of the fibres had been further degraded into fibrils (F) (Fig. 3C-E). Fibril formation in plant cell wall is believed to involve breakdown in the lignin cross linkages, degradation of cellulose crystals and shortening of cellulose chains [26, 27]. With the *B. akibai*+EM fermentation there were perforations of approx. 2-6 µm across in the cell walls, which were possibly micropores (MP) (Fig. 3B). Micropore formation in cassava parenchyma cell walls has been reported with microwave-heating and acid pre-treatment of the roots [28]. Micropore formation was attributed cell wall weakening due to hydrolysis of hemicellulose and lignin constituents within the cellulose-hemicellulose-lignin polymer matrix [28].

FTIR and WAXS of the Destarched Cassava CWM

Comparison of the average normalised IR spectra of CWM remaining after EM and *Bacillus* spp. fermentations showed differences at the 895 cm^{-1} wavenumber (Fig. 4A). The IR spectra shoulder was more pronounced in the *B. cellulosilyticus*+EM and *Bacillus* spp. in combination+EM treatments but less prominent in the EM, *B. akibai*+EM and *B. hemicellulosilyticus*+EM treatments. In contrast, at 1075 cm^{-1}, the peak vibration intensity was prominent in the spectra from the EM fermentation and less prominent in the *Bacillus* spp. fermentations. In contrast, at 1610 cm^{-1}, the vibrational peaks from the *Bacillus* spp. treatments were less pronounced compared to the EM. FTIR was used to study potato tuber parenchyma CWM and wavenumbers were assigned to pectin, xyloglucan and cellulose [29]. Accordingly, the IR spectra peak at 1075 cm^{-1} (C-O stretching, C-C stretching, xyloglucan ring) suggests that *B. cellulosilyticus*+EM and *Bacillus* spp. in combination+EM hydrolysed

more xyloglucans compared to the other fermentations. At 895 cm^{-1}, (C1-H bending, xyloglucan/cellulose β-anomeric link), the spectra data suggest *B. cellulosilyticus*+EM and *Bacillus* spp. in combination+EM caused more hydrolysis of non-cellulosic cell wall polymers compared to the others. Concomitantly, at 1610 cm^{-1} (COO^- antisymmetric stretching, polygalacturonic acid, carboxylate, pectin ester group), the data indicates less hydrolysis of cell wall pectin by the EM compared to the *Bacillus* spp. fermentations. These findings are consistent with the *Bacillus* spp. extracellular cellulase and polygalacturonase activities (Fig. 2). Furthermore, the findings agree with the GC data (Table 1B) that cassava fermentation using alkaliphilic *Bacillus* spp. primarily resulted in the loss of cell wall pectin and hemicellulose.

These apparent cell wall modifications ascribed by FTIR vibration peaks were investigated using WAXS. WAXS can provide a more accurate assessment of cell wall cellulose crystallinity than FTIR [30]. WAXS diffraction spectra of the CWMs from all the fermentations revealed three planes (denoted by small lines) at 2θ approx. 15.5°, 22.5° and 37.7°. The peak at 2θ approx. 22.5° has been assigned to the (002) plane of cellulose I [31]. All the *Bacillus* fermentations resulted in a significant ($p<0.05$) increase in CWM relative crystallinity compared to the EM fermentation (Fig. 4B). Furthermore, all *Bacillus* spp. fermentations resulted in increased peak (002) intensity compared to the EM treatment. An increase in diffraction peak (002) intensity is indicative of higher crystalline cellulose molecular chain regularity [32]. Thus, the cell wall degrading enzymes produced by *Bacillus* spp. fermentations seemed to have partially hydrolysed the amorphous fractions of the cell wall polysaccharides.

Effect of Alkaliphilic *Bacillus* spp. Fermentation on Starch Yield

Fermentation of the cassava using the alkaliphilic *Bacillus* spp. resulted in a very considerable reduction in the amount of the fibre-rich fraction, by 54-72% (Table 2). This is the waste fraction in cassava wet milling. It is thus significant that its starch content was reduced by 15-33% and the total weight of starch in this waste fraction was reduced by 63-79%. However, the *Bacillus spp.* fermentations significantly ($p < 0.05$) reduced the total amount of starch present in the starch- and fibre-rich fractions by up to 5%. This is presumably due to extracellular amylase activity exhibited by such fermentations [10]. Notwithstanding this, all the *Bacillus* spp fermentations resulted in considerably higher starch extraction yields (86.2-90.5%) than the EM fermentation (73.4%), with no significant difference ($p \geq 0.05$) in performance between the different *Bacillus* species.

Conclusions

Solid-state fermentation with alkaliphilic *Bacillus* spp. considerably weakens cassava root parenchyma cell walls, resulting greater cell wall disintegration during wet milling and consequently more release of starch granules, substantially improving starch extraction. The weakening of the cell walls is as result of action of alkaline-stable cell wall degrading enzymes produced by the *Bacillus* spp., in particular polygalacturonase, cellulase and probably xylanase. These enzymes hydrolysed the pectin, and hemicellulose fractions and to a lesser extent the cellulose. Therefore, solid state fermentation by alkaliphilic *Bacillus* spp. is a promising technology to improve the efficiency of cassava root cell wall disintegration during wet milling and hence increasing starch yield, without the use of commercial cell wall degrading enzymes or potentially polluting processing chemicals, such as strong acids or bases.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

References

1. Parmar, A., Sturm, B., & Hensel, O. (2017). Crops that feed the world: Production and improvement of cassava for food, feed, and industrial uses. *Food Security, 9*, 907-927.
2. Halley, P. J., & Avérous, L. (2014). *Starch polymers: From genetic engineering to green application*. San Diego, CA: Elsevier, (Chapter 3).
3. Saengchan, K., Nopharatana, M., Lerdlattaporn, R., & Songkasiri, W. (2015). Enhancement of starch-pulp separation in centrifugal-filtration process: Effects of particle size and variety of cassava root on free starch granule separation. *Food Bioproducts Processing, 95*, 208-217.
4. Sriroth, K., Piyachomkwan, K., Wanlapatit, S., & Oates, C. G. (2000). Cassava starch technology: the Thai experience. *Starch-Stärke, 52*, 439-449.
5. Ray, R. C., & Sivakumar, P. S. (2009). Traditional and novel fermented foods and beverages from tropical root and tuber crops: review. *International Journal of Food Science and Technology, 44*, 1073-1087.
6. Ngea, G. L. N., Guillon, F., Ngang, J. J. E., Bonnin, E., Bouchet, B., & Saulnier, L. (2016). Modification of cell wall polysaccharides during retting of cassava roots. *Food Chemistry, 213*, 402-409.

7. Adetunji, A. I., du Clou, H., Walford, S. N., & Taylor, J. R. N. (2016). Complementary effects of cell wall degrading enzymes together with lactic acid fermentation on cassava tuber cell wall breakdown. *Industial Crops and Products, 90*, 110-117.
8. Padonou, S. W., Nielsen, D. S., Akissoe, N. H., Hounhouigan, J. D., Nago, M. C., & Jakobsen, M. (2010). Development of starter culture for improved processing of Lafun, an African fermented cassava food product. *Journal of Applied Microbiology*, *109*, 402-1410.
9. Horikoshi, K. (1999). Alkaliphiles: Some applications of their products for biotechnology. *Microbiology and Molecular Biology Reviews, 63*, 735-750.
10. Nogi, Y., Takami, H., & Horikoshi, K. (2005). Characterization of alkaliphilic *Bacillus* strains used in industry: proposal of five novel species. *International Journal of Systematic and Evolutionary Microbiology, 55*, 2309-2315.
11. Fujinami, S., & Fujisawa, M. (2010). Industrial applications of alkaliphiles and their enzymes–past, present and future. *Environmental Technology, 31*, 845-856.
12. Odoch, M., Buys, E. M., & Taylor, J. R. N. (2017). Mechanism of cassava tuber cell wall weakening by dilute sodium hydroxide steeping. *Food Chemistry*, *228*, 338-347.
13. Gessesse, A., & Mamo, G. (1999). High-level xylanase production by an alkaliphilic *Bacillus* sp. by using solid state fermentation. *Enzyme and Microbial Technology, 25*, 68-72.
14. Amoa-Awua, W.K.A., & Jakobsen, M. (1995). The role of *Bacillus* species in the fermentation of cassava. *Journal of Applied Microbiology, 79*, 250-256.
15. Kumar, L., Kumar, D., Nagar, S., Gupta, R., Garg, N., Kuhad, R.C., & Gupta, V.K. (2014). Modulation of xylanase production from alkaliphilic *Bacillus pumilis* VLK-1 through process optimization and temperature shift operation. *3 Biotech*, *4*, 345-356.

16. Salvador, L. D., Suganuma, T., Kitahara, K., Tanoue, H., & Ichiki, M. (2000). Monosaccharide composition of sweetpotato fiber and cell wall polysaccharides from sweetpotato, cassava, and potato analyzed by the high-performance anion exchange chromatography with pulsed amperometric detection method. *Journal of Agricultural and Food Chemistry*, *48*, 3448-3454.
17. Ramírez-Tapias, Y. A., Rivero, C. W., Britos, C. N., & Trelles, J. A. (2015). Alkaline and thermostable polygalacturonase from *Streptomyces halstedii* ATCC 10897 with applications in waste waters. *Biocatalysis and Agricultural Biotechnology*, *4*, 221-228.
18. AACC. (2000). *Approved methods of the American Association of Cereal Chemists* (10th ed). St. Paul, MN: AACC, (Method 44-15A Moisture—Air-Oven).
19. Wokadala, O. C., Ray, S. S., & Emmambux, M. N. (2012). Occurrence of amylose–lipid complexes in teff and maize starch biphasic pastes. *Carbohydrate Polymers, 90*, 616-622.
20. Horikoshi, K. (1996). Alkaliphiles-from an industrial point of view. *FEMS Microbiology Reviews, 18*, 259-270.
21. Nyanga-Koumou, A. P., Ouoba, L. I. I., Kobawila, S. C., & Louembe, D. (2012). Response mechanisms of lactic acid bacteria to alkaline environments: a review. *Critical Reviews in Microbiology*, *38*, 185-190.
22. Jayani, R.S., Shukla, S.K., & Gupta, R. (2010). Screening of bacterial strains for polygalacturonase activity: its production by *Bacillus sphaericus* (MTCC 7542). *Enzyme Research*, 306785.
23. Mead, D., Drinkwater, C., & Brumm, P. J. (2013). Genomic and enzymatic results show *Bacillus cellulosilyticus* uses a novel set of LPXTA carbohydrases to hydrolyze polysaccharides. *PloS ONE, 8*, e 61131.

24. Staack, L., Della Pia, E.A., Jørgensen, B., Petersson, D., & Pedersen, N.R. (2019). Cassava cell wall characterization and degradation by a multicomponent NSP-targeting enzyme (NSPase). *Scientific Reports 9*:10150.
25. Figueiredo, P. G., Moraes-Dallaqua, M. A. D., Bicudo, S. J., Tanamati, F. Y., & Aguiar, E. B. (2015). Development of tuberous cassava roots under different tillage systems: descriptive anatomy. *Plant Production Science, 18*, 241-245.
26. Hoeger, I. C., Nair, S. S., Ragauskas, A. J., Deng, Y., Rojas, O. J., & Zhu, J. Y. (2013). Mechanical deconstruction of lignocellulose cell walls and their enzymatic saccharification. *Cellulose, 20*, 807-818.
27. Wang, Q. Q., Zhu, J. Y., Gleisner, R., Kuster, T. A., Baxa, U., & McNeil, S. E. (2012). Morphological development of cellulose fibrils of a bleached eucalyptus pulp by mechanical fibrillation. *Cellulose, 19*, 1631-1643.
28. Cheng, J., Lin, R., Ding, L., Song, W., Li, Y., Zhou, J., & Cen, K. (2015). Fermentative hydrogen and methane cogeneration from cassava residues: Effect of pretreatment on structural characterization and fermentation performance. *Bioresource Technology, 179*, 407-413.
29. Szymanska-Chargot, M., & Zdunek, A. (2013). Use of FT-IR spectra and PCA to the bulk characterization of cell wall residues of fruits and vegetables along a fraction process. *Food Biophysics, 8*, 29-42.
30. Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., & Johnson, D. K. (2010). Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for Biofuels, 3*, 1-10.
31. Leite, A. L. M. P., Zanon, C. D., & Menegalli, F. C. (2017). Isolation and characterization of cellulose nanofibers from cassava root bagasse and peelings. *Carbohydrate Polymers, 157*, 962-970.

32. Chen, W., Yu, H., Liu, Y., Chen, P., Zhang, M., & Hai, Y. (2011). Individualization of cellulose nanofibers from wood using high-intensity ultrasonication combined with chemical pretreatments. *Carbohydrate Polymers, 83*, 1804–1811.

Figure captions

Figure 1 Growth of endogenous microflora (EM) and alkaliphilic *Bacillus* spp. on ground cassava pieces (A) Growth, (B) pH change.

Error bars indicate standard deviation, n = 3.

Figure 2 Effect of endogenous microflora (EM) and alkaliphilic *Bacillus* spp. fermentation of ground cassava for 72 h on extracellular cell wall degrading enzyme production.

A: Cellulase activity - A1: Relative activity of the fermentations, A2: Activity in Viscozyme equivalents,

B: Endo-polygalacturonase activity - B1: Relative activity of the fermentations, B2: Activity in Pectinex equivalents.

Figure 3 Scanning electron microscopy showing the effect of endogenous microflora (EM) and alkaliphilic *Bacillus* spp. fermentation of ground cassava for 72 h on the root parenchyma cell walls; (A) EM, (B) *B. akiba* +EM, (C) *B. cellulosilyticus*+EM, (D) *B. hemicellulosilyticus*+EM and (E) *Bacillus* spp. in combination+EM.

MP = possible micropore in cell wall, CWF = cell wall fibres, F = Fibrils, RCM = ruptured cell wall fibre

Figure 4 ATR FTIR spectroscopy and WAXS X-ray diffraction showing the effect of endogenous microflora and alkaliphilic *Bacillus* spp. fermentation of ground cassava for 72 h on the cell wall material.

(A) Average normalised FTIR spectra (1800–400 cm^{-1}) of the CWMs from the different fermentation treatments.

(B) WAXS X-ray diffraction spectra of the CWMs from the different fermentation treatments.

Values in brackets are the mean percentage relative crystallinity for each fermentation treatment ± one SD, n = 2. Means with different letters are significantly different ($p<0.05$).

Table 1 Effect of endogenous microflora (EM) and alkaliphilic *Bacillus* spp. fermentation of ground cassava for 72 h on cell wall degradation and the sugar composition of the remaining cell wall material

(A) Cell wall disintegration

Fermentation type	**Fibre-rich fraction particle size distribution (%)**			**Cell wall material remaining**
	> 250 µm	**≤ 250 µm to > 106 µm**	**≤ 106 µm to > 25 µm**	**(g/100 g fresh cassava root)**
EM	91.9^{c}±0.9	8.1^{a}±0.9	0.0^{a}±0.0	2.7^{c}±0.11[1]
B. akibai+EM	75.3^{bc}±6.1 (-18.1%)	20.1^{ab}±5.2 (+148.1%)	4.7^{a}±1.7 (+470%)	1.7^{b}±0.2 (-37.0%)[2]
B. cellulosilyticus+EM	45.0^{a}±19.9 (-51.0%)	43.3^{b}±25.7 (+434.6%)	11.7^{ab}±6.5 (+1170%)	1.5^{a}±0.0 (-44.4%)
B. hemicellulosilyticus+EM	42.1^{a}±14.5 (-54.2%)	22.5^{ab}±11.3 (+177.8%)	35.4^{c}±25.7 (+3540%)	1.5^{ab}±0.1 (-44.4%)
B. spp. in combination+EM	57.7^{ab}±22.2 (-37.4%)	9.9^{a}±15.6 (+22.2%)	32.4^{bc}±9.6 (+3240%)	1.4^{a}±0.0 (-48.1%)

[1]Means± standard deviations of three independent experiments

Means of values in the same column but with different letters are significantly different ($p<0.05$)

[2]Values in parentheses for each analysis are percentage differences between the means of endogenous microflora and *Bacillus* spp. treatments

(B) Sugar composition (mg/100 g) of the remaining cell wall material

Fermentation type	**Rhamnose**	**Fucose**	**Arabinose**	**Xylose**	**Mannose**
EM	5.3[b]±0.3[1]	10.3[b]±2.3	54.0[c]±2.3	134.8[b]±15.1	32.7[b]±4.8
B. akabai+EM	3.7[a]±0.7 (-30.2%)[2]	6.2[a]±0.0 (-39.8%)	23.6[b]±1.4 (-56.3%)	95.8[a]±8.2 (-28.9%)	20.2[a]±1.6 (-38.2%)
	[1.6][3]	[4.1]	[30.4]	[39.0]	[12.5]
B. cellulosilyticus+EM	2.8[a]±0.6 (-46.8%)	7.5[a]±0.7 (-27.7%)	18.5[a]±1.9 (-65.7%)	94.8[a]±2.1 (-29.7%)	20.7[a]±1.6 (-36.9%)
	[2.5]	[2.8]	[35.5]	[40.0]	[12.0]
B. hemicellulosilyticus+EM	2.5[a]±0.8 (-52.8%)	7.1[a]±0.6 (-30.8%)	17.9[a]±0.0 (-66.8%)	87.0[a]±3.9 (-35.4%)	20.1[a]±0.6 (-38.5%)
	[2.8]	[7.2]	[36.1]	[47.8]	[12.6]
B. spp. in combination+EM	2.9[a]±0.2 (-45.7%)	6.7[a]±0.3 (-35.2%)	20.1[ab]±0.7 (-62.7%)	83.9[a]±2.9 (-37.8%)	17.2[a]±0.4 (-47.5%)
	[2.4]	[3.6]	[33.9]	[50.9]	[15.5]

Fermentation type	Galactose	Glucose	Galacturonic acid	Total NSP sugars	NSP sugar recovery
EM	68.1^{c}±4.4	602.4^{b}±50.2	108.1^{c}±2.6	1015.8^{b}±82.0	38.3^{a}±1.3
B. akabai+EM	38.1^{b}±0.2 (-44.1%)	438.4^{a}±50.5 (-27.2%)	76.5^{b}±6.4 (-29.3%)	702.6^{a}±66.3 (-30.8%)	40.6^{ab}±0.2 (+6.0%)
	[30.0]	[164.0]	[31.6]	[313.2]	
B. cellulosilyticus+EM	32.8^{ab}±0.2 (-51.9%)	433.8^{a}±4.6 (-28.0%)	66.8^{a}±2.5 (-38.2%)	677.7^{a}±7.8 (-33.3%)	46.4^{c}±0.5 (+21.1%)
	[35.3]	[168.6]	[41.3]	[338.1]	
B. hemicellulosilyticus+EM	32.6^{a}±1.5 (-52.2%)	397.8^{a}±10.2 (-34.0%)	68.4^{ab}±2.5 (-36.8%)	633.5^{a}±17.7 (-37.6%)	41.0^{b}±0.5 (+7.0%)
	[35.5]	[204.6]	[39.7]	[382.5]	
B. spp. in combination+EM	33.7^{ab}±1.3 (-50.5%)	363.2^{a}±2.3 (-39.7%)	65.6^{a}±0.8 (-39.3%)	593.2^{a}±1.2 (-41.6%)	42.2^{b}±1.4 (+10.2%)
	[34.4]	[239.2]	[42.5]	[422.6]	

[1]Means± standard deviations of three independent experiments

Means of values in the same column but with different letters are significantly different ($p<0.05$)

[2]Values in curved brackets for each sugar are percentage differences between the means of endogenous microflora and *Bacillus* spp. treatments

[3]Values in square brackets for each sugar are quantitative differences between the means of endogenous microflora and *Bacillus* spp. treatments

Table 2 Effect of endogenous microflora (EM) and alkaliphilic *Bacillus* spp. fermentation for 72 h on starch extraction yield from cassava pieces wet milled using a 2 mm screen opening size (yield/kg cassava roots)

Fermentation type	Starch-rich fraction weight (g dry basis)	Starch-rich fraction purity (% starch)	Starch-rich fraction Weight of starch (g)	Starch yield (%)
EM	223.5^{a}±1.0[1]	89.9^{a}±2.1	201.0^{a}±5.6	73.4^{a}±1.2
B. akibai+EM	267.8^{b}±7.9 (+19.8%)[2]	90.3^{a}±1.4 (+0.4%)	241.8^{bc}±10.4 (+20.3%)	88.3^{bc}±3.8 (+20.3%)
B. cellulosilyticus+EM	278.9^{c}±1.2 (+24.8%)	88.9^{ab}±0.8 (-1.1%)	247.9^{c}±3.2 (+23.3%)	90.5^{c}±1.2 (+23.3%)
B. hemicellulosilyticus +EM	269.8^{b}±4.0 (+20.7%)	87.6^{ab}±1.0 (-2.6%)	236.3^{b}±4.6 (+17.6%)	86.2^{b}±1.7 (+17.4%)
B. spp. in combination+EM	283.9^{c}±1.6 (+27.0%)	86.8^{b}±1.9 (-3.5%)	246.5^{bc}±4.5 (+22.6%)	90.0^{bc}±1.6 (+22.6%)

Fermentation type	Fibre-rich fraction weight (g dry basis)	Fibre-rich fraction (% starch)	Fibre-rich fraction Weight of starch (g)	Weight of starch in both fractions (g)
EM	98.0^{c}±0.9	74.5^{c}±3.4	73.0^{c}±3.4	273.9^{c}±5.4
B. akibai+EM	45.0^{b}±8.3 (-54.1%)	60.6^{b}±3.9 (-18.7%)	27.4^{b}±6.2 (-62.5%)	269.2^{bc}±4.3 (-1.7%)
B. cellulosilyticus+EM	30.2^{a}±0.9 (-69.2%)	50.8^{a}±2.6 (-31.8%)	15.4^{a}±1.2 (-78.9%)	263.2^{ab}±3.8 (-3.9%)
B. hemicellulosilyticus+EM	40.0^{b}±2.0 (-59.2%)	63.7^{b}±0.2 (-14.5%)	25.5^{b}±1.3 (-65.1%)	261.7^{ab}±4.0 (-4.4%)
B. spp. in combination+EM	27.5^{a}±0.3 (-71.9%)	49.8^{a}±1.9 (-33.2%)	13.7^{a}±0.4 (-81.2%)	260.2^{a}±4.9 (-5.0%)

[1]Means± standard deviations of three independent experiments

Mean values in the same column within a block with different letters are significantly different ($p<0.05$)

[2]Values in parentheses for each analysis are percentage differences between the means of endogenous microflora and *Bacillus* spp. fermentations

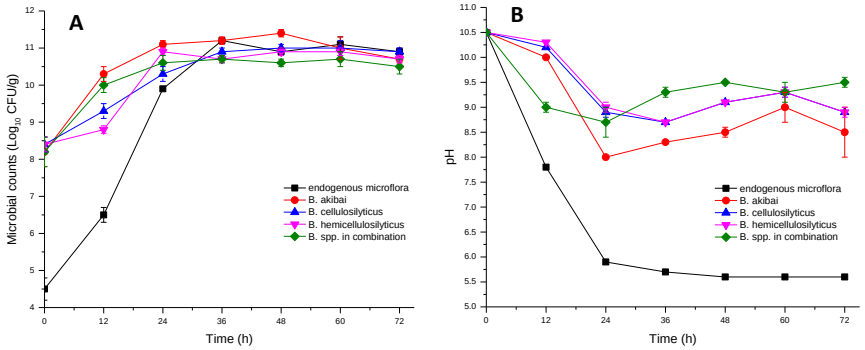
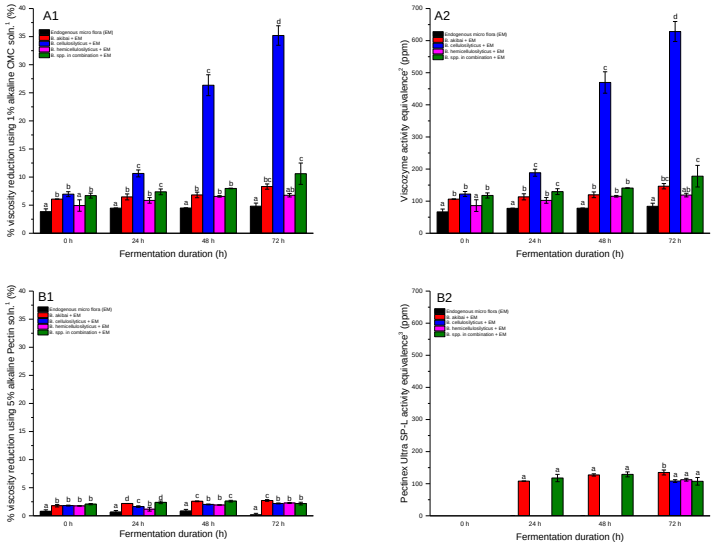

A
Microbial counts (Log10 CFU/g)
Time (h)
endogenous microflora
B. akibai
B. cellulosilyticus
B. hemicellulosilyticus
B. spp. in combination
B
pH
Time (h)
endogenous microflora
B. akibai
B. cellulosilyticus
B. hemicellulosilyticus
B. spp. in combination

Figure1



[1]Means±standard deviations of three independent experiments
[2]Regression between % viscosity reduction and Viscozyme L activity equiv.; y = 0.0558x + 2.42, R^2 = 0.98
[3]Regression between % viscosity reduction and Pectinex Ultra SP-L activity equiv.; y = 0.0199x + 2.09, R^2 = 0.95. For % viscosity reduction less than 2.09 (standard curve y-intercept, enzyme blank) polygalacturonase activity was regarded as not detectable.
Means of values in the same fermentation duration but with different letters are significantly different ($p<0.05$)

Figure2

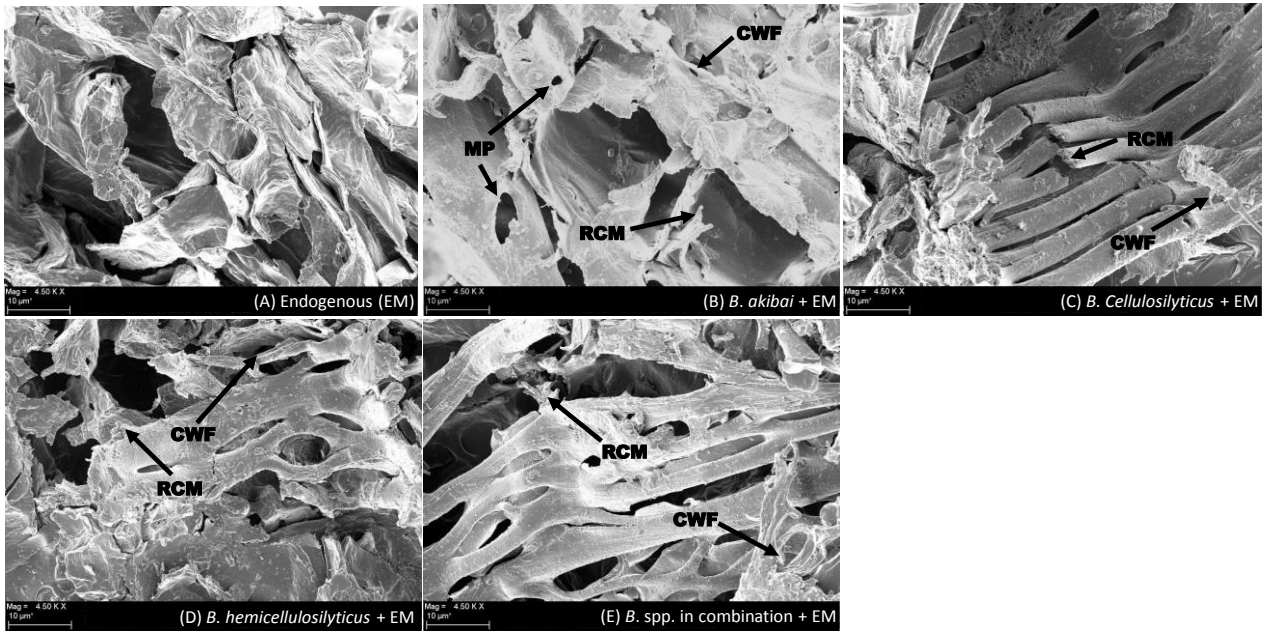

(A) Endogenous (EM)
CWF
MP
RCM
(B) B. akibai + EM
RCM
CWF
(C) B. Cellulosilyticus + EM
CWF
RCM
(D) B. hemicellulosilyticus + EM
RCM
CWF
(E) B. spp. in combination + EM

Figure 3

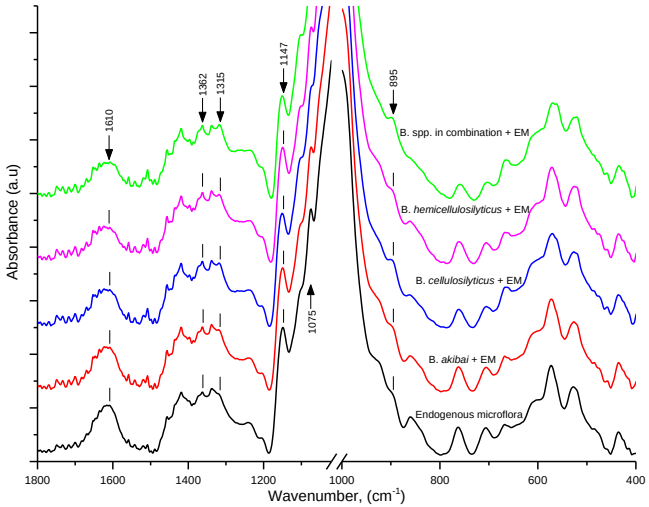

1610
1362
1315
1147
1075
895
B. spp. in combination + EM
B. hemicellulosilyticus + EM
B. cellulosilyticus + EM
B. akibai + EM
Endogenous microflora
Absorbance (a.u)
1800
1600
1400
1200
1000
800
600
400
Wavenumber, (cm-1)

Figure 4A

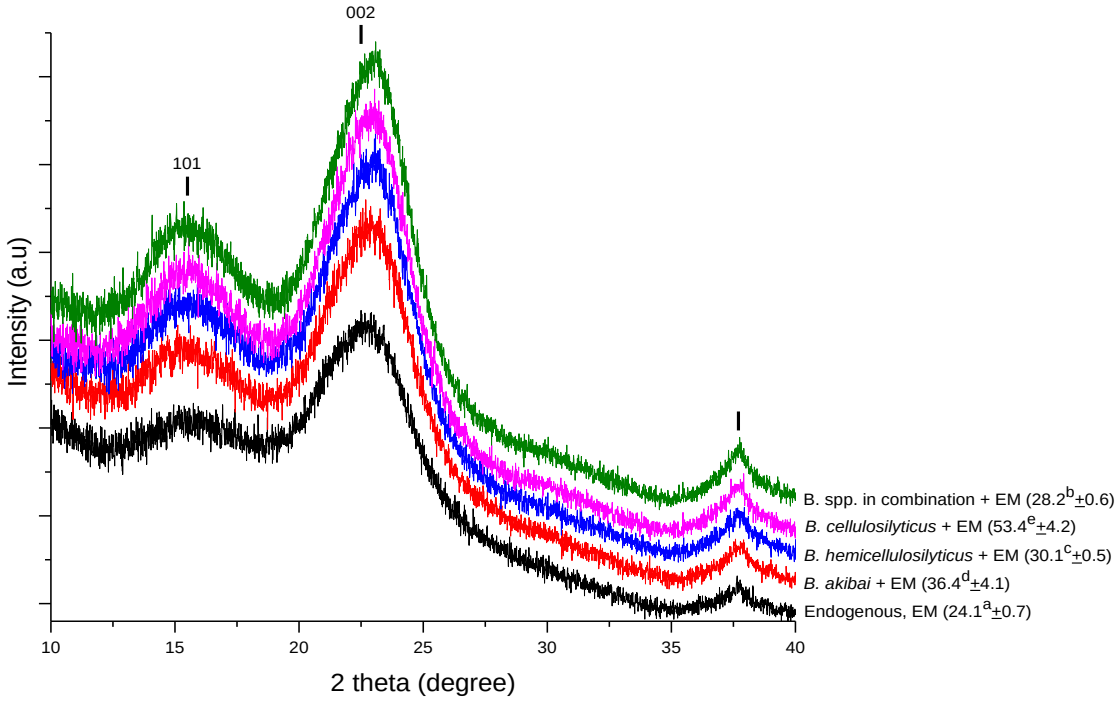

002
101
Intensity (a.u)
10
15
20
25
30
35
40
2 theta (degree)
B. spp. in combination + EM (28.2b±0.6)
B. cellulosilyticus + EM (53.4e±4.2)
B. hemicellulosilyticus + EM (30.1c±0.5)
B. akibai + EM (36.4d±4.1)
Endogenous, EM (24.1a±0.7)

Figure 4B