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Production of Hydrogen, Ethanol and Organic Acids from Stale Bread Using Mixed Yeasts-Bacteria Microbial Consortia

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One of the main methods for obtaining mixed fermentative acidogenic inocula is the isolation of spore-forming bacteria (SFB) from different mixed matrices. SFB have proven to be suitable inocula in Dark Fermentation systems, although they have certain limitations. In the present study, the formulation of a mixed consortium is studied, by combining SFB and Water Kefir (WK) in Mixed-Yeast-bacteria (MYB) fermentations tests. First, both inocula are properly selected and maintained. Then, a scan of different pretreatments is performed on stale bread that was selected as retrieved substrate for the MYB tests. MYB tests are conducted in batch mode, under anaerobic conditions, using the basic-pretreated stale bread. Systems inoculated with independent inocula (either SFB and WK, in a 10% *v/v*) and combined (WK+SPB, 5+5% *v/v*) are studied in a three-cycles compatibility experimental campaign. The gas production and yield, its composition and the liquid titers of organic acids and ethanol are measured to shed light on the fermentation dynamics in each case. The adaptation cycles resulted in progressively reduced lag phases for all systems, and interesting results were obtained for the WK+SFB system, which exhibited higher hydrolytic activity and achieved the highest hydrogen, ethanol and organic acid productivity.

1. Introduction

In recent years, the increase in environmental problems has intensified the search of sustainable solutions to meet societal needs. Biorefinery approaches indeed currently seek alternative pathways, primarily by exploiting biomass renewable resources, for the production of useful chemicals and (bio)fuels. Most biorefinery processes can be classified under the thermo or the bio-based axis. The difference lies in the principle that is exploited for the activation and conversion of biomass; each of them requires a different technology chain (i.e., upstream operations, conversion processes and downstream steps up to final purification if it is required).
A wide range of biotechnological processes have been proposed in the literature for the production of energy carriers and chemical compounds of interest. The most consolidated applications to valorize the organic matter (OM) are the aerobic and the anaerobic treatment of OM (wastewaters, refuses, activated sludges, etc.) and composting. These biotechnological processes exploit the vast diversity of microbial consortia instead of monocultures due to the complexity of the system; but they provide key services such as for water sanitation, energy recovery alongside with nutrient recycling by producing organic soil conditioners useful for the agricultural sector. The Dark Fermentation (DF) has been widely studied for organic residues; the process can be considered a truncated version of anaerobic digestion (AD), resulting in the production of biohydrogen in the gaseous phase and short-chain carboxylic acids in the liquid phase and eventually solvents (mainly ethanol and butanol). The DF alone has not yet reached consolidated presence in biorefineries environments, since the energy sustainability of the process can be hardly reached. However, DF has been candidated as key processing unit within multistep technological chains, where DF serve as biological pre-treatment for the hydrolysis and first fermentative cycles (i.e., acidogenesis and acetogenesis), for example in two step AD (increasing the total energy produced as gas phase CH_4+H_2) (Ruggeri et al., 2015).

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The biotic phase in DF consists of fermentative bacteria, being the Firmicutes phylum (i.e., the anaerobic branch) the main responsible (Gómez-Camacho et al., 2021). Hence, the selection of spore-forming microorganisms in different environments has been useful for the preparation of DF inocula. But these consortia typically require pH control (due to the high organic acids production) and degassing to achieve high productivities, and ethanol is produced at rather low titers (Ruggeri et al., 2015). Indeed, bioethanol (as energy carrier) is mainly produced from agricultural feedstocks (e.g., corn, sugarcane, sorghum), after intensive pre-treatments and downstream separations, using selected *Saccharomyces cerevisiae* strains. However, bioethanol production presents two critical bottlenecks: first the EROI (Energy Return On Investment) analysis has shown poor or questionable energy sustainability (Hall et al., 2011), and second, the *food-fuel* conflict it is not solved.
An interesting environmental matrix that can be exploited for engineering microbial communities (Gómez-Camacho and Ruggeri, 2020) is water kefir (WK), since its ecological diversity includes mixed lactic acid bacteria, acetic acid bacteria and yeasts (i.e., mainly Firmicutes, Actinomycetota, Proteobacteria bacteria and Ascomycota fungi). WK is mainly used in home (probiotic) fermentations, under facultative aerobic conditions and fed with a wide-range of substrates, although the robustness of the microbic community serves as a study-model and as a platform to select microbial groups of interest (due to its high metabolic plasticity). The division of labor or functional specialization in WK fermentations seems to rely in bacteria-yeasts interactions: while bacteria are responsible for the hydrolysis and the first acidogenic conversion of solubilized substrates, yeasts are mainly fed with the produced fermentable sugars for the production of ethanol. In turn, ethanol production can buffer the system avoiding low acidic conditions, but it can also cross-feed bacteria via (metabolic) ethanol oxidation reactions (Melkonian et al., 2019).
It has been widely hypothesized in the literature that mixed yeasts-bacteria consortia could be a suitable catalyst for biorefinery process aiming at the simultaneous production of ethanol, organic acids and hydrogen (Dionisi and Silva, 2016). Ideally, by tuning high hydrolytic and acidogenic/solventogenic capabilities within stable yeasts-bacteria consortia, the energy intensive pre-treatments required for the sustainable production of ethanol can be reduced (i.e., as energy carrier), especially from retrieved biomasses.
In this work, preliminary compatibility tests for formulating a MYB inoculum are performed. First, two indigenous sources of inoculum are selected and maintained, which contained mixed yeasts and bacteria: a home culture of WK and SPB selected from fresh cattle manure. The MYB are performed using a retrieved substrate (stale bread), after an alkaline pretreatment. The MYB tests are carried out under anaerobic conditions, at high substrate concentration, and using an inoculum ratio of 10% *v/v*. Both inocula (SFB and WK) were tested separately and in combination (WK+SFB) to observe synergistic effects. The production and composition of gas were monitored online, while the liquid phase was analyzed at the end of the MYB (organic acids and ethanol).

2. Materials and Methods

2.1 Inocula

Water Kefir grains and Water Kefir broth

Household water kefir grains were obtained from a local donor in the city of Turin (WKG2G0) and kept under refrigerated conditions (Laboratorio Biotecnologico - Politecnico di Torino, Turin, Italy). A fraction of these grains (20 g) was cultivated through a series of 8 consecutive backslopping in a bioreactor (Minifors I Infors HT, Bottmingen, Switzerland), using a sugar medium at 50 g/L (i.e., 50 % *w/w* of glucose and 50 % *w/w* of commercial sugar cane), low mesophilic conditions (22 °C) and an initial pH in the 5.5-6.0 range. These conditions were chosen based on literature references and preliminary tests aiming at enriching the broth in biomass, particularly yeasts. Continuous microscopic observations (BH-2 Olympus Italia Srl, Segrate, Italy) were performed to get insights into the biotic phase (i.e., planktonic cells in the liquid phase and entrapped cells within kefir grains) and their qualitative abundance in the liquid phase.

Spore-forming Bacteria (SFB) Inoculum

Indigenous spore-forming bacteria (SFB) were selected from cattle manure. First, a slurry was produced by diluting 200 g of manure with distilled water in a 1:1 *w/w* ratio. The obtained slurry was mixed for 2 h at 250 rpm in order to stress anaerobic SFB and initiate the suppression of the anaerobic methanogenic activity. After the homogenization and aeration step, the slurry was sequentially filtered three times using a 2 mm mesh sieve and afterwards using a cellulose filter. After the filtration step that removed large debris and clumps, SFB from the mixed culture were isolated by treating the filtrate with ethanol (55 % *v/v*) for 24 h in order to promote the sporulation of indigenous SFB (Koransky et al., 1978).

2.2 Substrate and pretreatments

The substrate for the fermentation tests was stale commercial ground bread (Buongiorno Natura, Venice, Italy). Due to the complex and starchy nature of this matrix, different pre-treatments were tested in order to scan for suitable options. Five different types of pretreatments at room temperature were performed: (BP) basic pretreatment (0.1 M NaOH); (AP) acid pretreatment (1.0 HCL); (SP) salt pretreatment (1.0 M $CaCl_2$); (EP* and EP**) enzymatic mix (Solgar Italia Spa, Padova, Italy) pretreatment at different concentration (1.0 and 10.0 % w_{enz}/w_{TS}); (ESP) enzymatic mix plus salt (ESP) (1 % w_{enz}/w_{TS} + 1.0 M $CaCl_2$) pretreatment. After the pre-treatments, aliquots of 8 mL were centrifuged at 4000 rpm for 30 min (IEC CL30 Thermo Scientific, Rodano, Italy) and the supernatant was filtered through 0.45 µm filters. The effect of the pretreatment was evaluated using absorbance readings at 440 nm and the Brix degrees (°Bx); while the supernatant and the centrifugation residue were morphologically analyzed through optical microscopy. After the initial pretreatment scan, high performance liquid chromatography (HPLC) analysis (see below) were performed on the raw and BP samples. Prior to the fermentation tests, the substrate was prepared (concentration: 100 g_{TS}/L) and it was treated with powdered NaOH up to pH=12, for 24 h; afterwards the pH was readjusted to pH=7 using a 10 M HCL solution.

2.3 Mixed Yeasts-Bacteria (MYB) fermentation tests

MYB fermentation tests were performed using a AMPTS II unit (Bioprocess Control AB, Lund, Sweden); three consecutive cycles were performed. The inocula for the MYB fermentation tests were: water kefir broth (WK), and the isolated SFB from cattle manure. These inocula are tested separately (WK, SFB) and in combination (WK+SFB). The batch MYB tests were conducted using 500 mL serum bottles with a working volume of 220 mL. After dosing the substrate (c. 200 mL of BP substrate) and the corresponding inoculum for each case (10 % *v/v* for the SFB and WK, and 5+5 % *v/v* for the WK+SFB), the bottles were flushed with nitrogen gas for 4 min to displace oxygen and reach anaerobic conditions; the bottles were then connected to the absorption and the gas measurement units of the AMPTS II. Four replicates for each tested inoculum are performed in each cycle, two were connected to gas absorption unit containing acidic water (pH=2.5-3.0) to allow the total produced gas to be recorded (H_2+CO_2), while the other two were connected to gas absorption bottles containing 3 M NaOH (i.e., CO_2 entrapment) for hydrogen production recording. The agitation of the systems was 150 rpm with on/off intervals of 5 minutes. The gas production was monitored online using the web server of the AMPTS II unit, and the tests were stopped when the plateau of the gas production was reached (c. 48 h).

2.4 Analytical measurements

Proximate analysis

The raw substrate, the initial medium and the fermented broths were characterized by means of a proximate analysis, for the estimation of the moisture content (MC), the total solids (TS) and the volatile solids (VS), and the ash fraction (AF) according to the standard APHA method. Gravimetric measurements were made using an analytical balance (E42S Gibertini Elettronica, Novate Milanese, Italy).

High-Performance Liquid Chromatography (HPLC)

A HPLC method was developed for the concomitantly quantification of key hydrolysis carbohydrates and fermentation products (i.e., maltose, organic acids and ethanol). The samples were first centrifuged at 4,000 rpm for 20 min, then the supernatants were filtered with 0.45 µm cellulose ester filters. The HPLC system consisted in an HPLC pump (422 Kontron Instruments, Milan, Italy), a refractive index detector (RDI) (RefractoMonitor IV Milton Roy, Riviera Beach, United States) and a size exclusion/ligand exchange column (Aminex HPX-87H Bio-rad, California, United States). The column was operated at 55 °C, under isocratic conditions using 5 mM H_2SO_4 as eluent at a rate of 0.8 mL/min.

pH, titratable acidity (TA), degree Brix (°Bx), absorbance measurements

The pH measurements were performed using a pH-meter (Micro-pH 2001 Crison Instruments SA, Barcelona, Spain). The degree Brix (°Bx) were measured using a Brix refractometer (LAISS Apparecchiature Scientifiche, Torino, Italy), by placing a drop of centrifuged and filtered samples into the reading chamber of the refractometer. Titratable acidity (TA) measurements were conducted on 5 mL of the raw and fermented samples (diluted 1:5 *v/v* with distilled water) using a standardized 0.1 N NaOH solution as titrant, and phenolphthalein (c. 100 µL of a 60% *v/v* ethanol solution) as visual endpoint indicator under constant mixing conditions (TA is expressed as eq. grams acetic acid, $g_{AA,eq}$/L). The absorbance measurements were performed using a UV/VIS spectrophotometer (PerkinElmer, Waltham, USA); samples were centrifugated at 4,000 rpm for 30 minutes, filtered with a 0.45 µm cellulose ester filter and properly diluted to collect the readings.

3. Results

3.1 Inocula

Water Kefir grains and Water Kefir broth

The first studied source of inoculum was water kefir grains. Typically, kefir grains are used as inoculum source in backslopping cultivations, the resulting broth (the kefir broth) is the desired product in homemade probiotics cultivations. A similar procedure was followed then for the maintenance of the grains (WKG) and to obtain biomass-enriched broths (WK) that could serve for the latter MYB fermentation tests. While the first broths resulting from the backslopping cultivations were poor in biomass (predominantly bacterial biomass, Figure 1a), an enrichment in biomass was observed as the backsloppings progressed (Figure 1b), especially with respect to the yeasts. In fact, microscopic observations revealed that although initial broths appeared to contain few (vegetative) yeast cells, the WKG contained a significant amount in both, the true septate and pseudohyphae forms (Figure 1c).

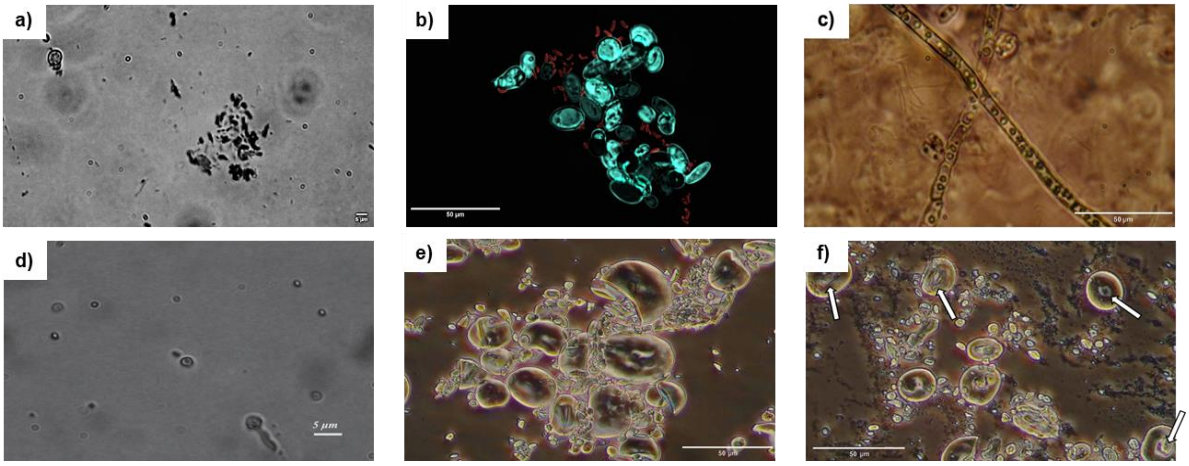

Figure 1. Micrographs of (a) BS1 WKG broth, (b) BS8 WKG broth, (c) focus-stacked m. of a WKG, (d) SFB inoculum, substrate (e) before the pre-treatment, (f) 24 h NaOH-treated.

The backslopping cultivations proved consistent for all batches. That is, acidification occurred in all systems typically starting from pH=5.5-7.0 values and finishing around pH=2.5-3.5 (although, BS 7 and BS 8 started from pH=3.2 probably due to cumulative acidification of the grains) (Figure 2a). Similarly, the final TA of each BS lied around 0.96-1.32 g AA_{eq}/L, except from BS 7 which reached 1.90 g AA_{eq}/L (Figure 2b). Another key parameter, the ORP also served to monitor the state of BS systems. During the first four BS, the ORP was predominantly in the aerobic range (i.e., 150-320 mV), while for the last four BS the dynamics of the culture switched towards more anaerobic potentials (finishing around -100 to 0 mV)(Figure 2c). It was decided to use the latter system (BS 8) as inoculum in the MYB tests (i.e., termed WK) since this bioreactor culture method (under open conditions and using combined sugars) gave consistent results in terms of the obtained trends among the BS.

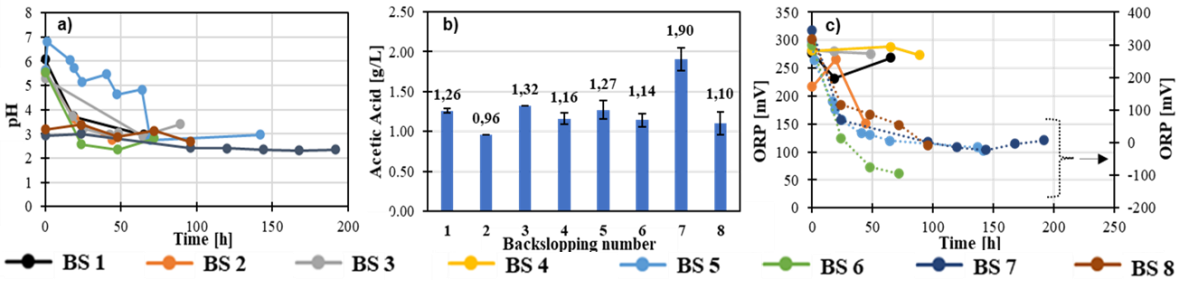

Figure 2. Measured parameters in the 8 backslopping of the WK inoculum maintenance: (a) pH, (b) final TA, and (c) ORP evolution.

Spore-forming Bacteria (SFB) Inoculum

In the literature there are a variety of methods for the selection of spore-forming bacteria (SPB) from mixed cultures, especially from bovine manure. SPB are selected in the context of biorefineries for their potential as Hydrogen-Forming Bacteria, which has already been demonstrated in DF, either by inoculating batch or

continuous systems (Ruggeri et al., 2015). Starting from cattle manure, the preparation of the slurry in aerated conditions first allowed the inhibition of the methanogenic population, while the treatment at high concentrations of ethanol promotes proteins denaturation and the (partial) dissolution of bacterial cell membranes, which ultimately leads to bacteria cell deaths. However, in mixed consortia where microorganisms belonging to the Firmicutes phylum are present, viable spores (Gómez-Camacho et al., 2021) can withstand high chemicals (including ethanol) concentrations. The resulting spores (SFB, Figure 1d) were used for the MYB tests.

3.2 Substrate and pretreatments

The pretreatment steps aimed at (partially) destroying the spatially complex structures of substrate matrix, in order to reduce lag time in MYB fermentations and to favor the availability of the substrate to microorganisms. The results (Table 1) indicated that BP and AP pre-treatments resulted in similar °Bx and A_{440} measurements, higher than the others tested pre-treatments methods (SP, EP, ESP). For the SP, EP, ESP methods, either lower °Bx or A_{440} were found. An outlier °Bx reading was collected for the SP systems, probably due to the interference with salty solution. Although the EP** resulted in high °Bx, the A_{440} resulted much lower compared to the AP and BP. The BP was then selected to treat the substrate before the MYB fermentations tests. Additionally, microscopy analyses were performed to get insights into the alkaline attack mechanisms (BP) on the stale bread matrix. In the raw substrate, whole starch gains could be appreciated (Figure 1e), and after the basic pretreatment, swelling of the starch granules and mechanical fracture in the central part of them (Figure 1f) was observed, as well as mixed residues in the suspension. This basic chemical attack on starch has also been reported in the literature for similar matrices (Uthumporn et al., 2012).

Table 1. °Br and absorbance measurement of different pre-treatments.

Pre-treatment	Raw substrate	BP	AP	SP	EP*	EP**	ESP
Sugars [°Bx]	0.6±0.1	1.0±0.1	0.8±0.1	5.8±0.1	0.4±0.1	0.9±0.1	0.5±0.1
$A_{440}\cdot10^3$ [AU]	26±1	97±1	96±1	58± 1	9±2	41±8	12±10

3.3 Mixed Yeast-Bacteria (MYB) fermentation tests

For the MYB tests, the previously selected and maintained inocula (see section 3.1) (i.e., WK and SFB) were tested separately and together to assess the effects of a mixed inoculum (WK+SFB). Three cycles were carried out to adapt each inoculum to the alkali-treated substrate. Gas evolution in anaerobic fermentative systems is a key *probe parameter* which can be used to infer the growth dynamics of mixed cultures, particularly at the early development stage for formulating inocula. Throughout the adaptation cycles, it was observed that the lag phase for gas production progressively decreases for all the tested inocula (Figure 3). Similarly, the accumulated gas production also increased from the first cycle to the second, while the third cycle resulted in lower yields for the WK and the mixed inoculum (WK+SFB). For the SFB, gas production further increased in the third cycle. This latter fact could be due to the refrigerated storage lapse between the second and third cycle, which was longer than the first one. Since WK and WK+SFB also contain a large proportion of yeasts during storage, the community composition could have been modified and the effects were reflected as lower gas production.

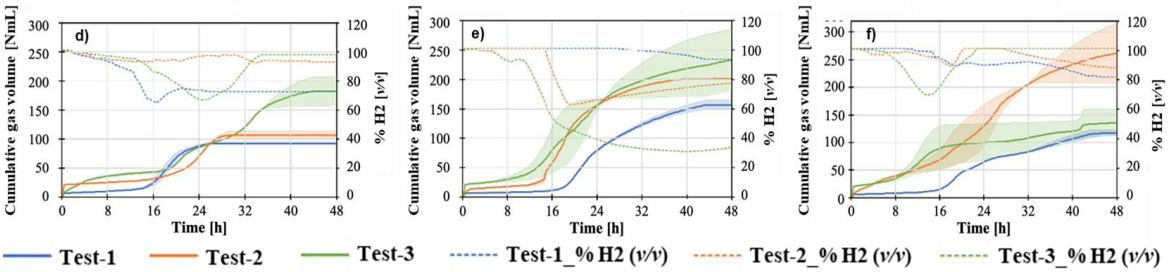

Figure 3. Gas evolution and composition during the three adaption cycles for a) WK; b) SFB; and c) WK+SFB.

The SFB inoculum was able to maintain (and progressively increase) gas production capabilities. These gas production curves suggested the presence of a well-conformed fermentative microbial community, showing latent, exponential production, and final stationary phases. For the WK, well defined gas production curves were obtained during the first and third cycles; while in the second the exponential phase was not well defined. This behavior is typically associated to configurational changes within microbial communities (either to adapt its hydrolytic capacities maintaining the populations abundance or to change the relative abundance of microbial groups within the consortia). The combined inoculum (WK+SFB) showed gas evolution curves with less pronounced exponential phases, probably due to the compatibility process of the two mixed consortia.

After the third adaptation cycle (Table 2), the SFB system achieved the higher gas production (251.50 mL), WK achieved (114.83 mL), while the mixed inoculum resulted in an intermediate value between them (170.01 mL). The composition of the gas phase in SFB systems reached 33.40 % *v/v* of H_2, while the WK and WK+SFB systems was higher than 95.00 % *v/v*. Hence, the higher hydrogen yields (8.09 L_{H2}/kg_{TS0}) were attained for the mixed inocula. An initial maltose concentration of 1.43 *w/v* % was measured, and in the final MYB broths, the maltose concentration was slightly higher for the WK system (+ 7 %), 250 % higher for the SFB and almost 300 % more for the WK+SFB system. This fact might indicate that the mixed system presented enhanced hydrolytic capabilities compared to the independent inocula, based on the further degradation of the (partially) basic-hydrolyzed starch residues into maltose. Significant differences were also encountered in the liquid metabolic products for each system (Table 2). After the third adaptation cycle, for the WK system, mainly lactic acid and ethanol were detected, while for the SFB the predominant titters correspond to lactic acid (twice as much as the WK) and ethanol (almost 8-fold increase compared to WK). Interestingly, the combined system exhibited the higher metabolic activity, presenting similar titters of lactic acid compared to the SFB, higher acetic acid compared to the WK (27 % increase) and the greatest ethanol production, achieving 2.45 g/L.

Table 2. Gas and liquid phase products of the three formulated inocula (III cycle MYB).

Inocula	V_{cum}	% H_2	$Y_{P/S}$	ΔTS	λ	Maltose	LA	AA	EtOH
	[mL]	[*v/v*]	[mL_{H2}/g_{TS0}]	[%]	[h]	[g/L]	[g/L]	[g/L]	[g/L]
WK	114.83±9.90	96.62±4.91	5.5±0.9	6.00±0.02	5.48±0.71	15.4±1.1	1.20±0.36	18.48±3.88	0.24±0.15
SFB	251.50±72.13	33.40±31.22	4.2±1.1	8.00±0.04	5.89±0.21	38.0±3.1	2.88±0.72	n.d.	1.82±1.34
WK+SFB	170.01±10.39	95.44±20.54	8.1±0.8	11.00±0.04	4.16±0.54	41.6±2.3	2.88±1.44	23.52±12.18	2.45±1.26

LA: lactic acid; AA: acetic acid; EtOH: ethanol.

4. Conclusions

Inocula compatibility tests were performed by mixing two indigenous sources, consisting in Spore-Forming Bacteria (SFB) and Water Kefir (WK). These inocula were first properly selected and maintained, and then were tested to ascertain the synergistic effects (SFB+WK) in mixed yeasts-bacteria batch anaerobic fermentation tests using a residual biomass (stale bread) as substrate. The basic pre-treatment was applied on the substrate and during the inocula compatibility cycles, a decreasing lag phase and increasing gas evolution were observed. Hydrolytic activity was encountered in all systems, but the (SFB+WK) system resulted in higher maltose concentrations compared to the others, as well as the highest hydrogen, ethanol and organic acid productivities. Hence, it is possible conclude that WK+SFB consortia could be a promising inoculum for MYB tests. Although, this type of compatibility tests is limited by the lack of pH control and quantitative microbial groups identification, it sheds light on the initial performance of the systems. Further investigations should asses the effects of the mixing ratio between inocula (here only 1:1 *v/v* was tested), test lower substrate loads or manipulate the consortia to tune the metabolism towards the desired products, as well as to investigate the fermentation dynamics for scaling-up purposes.

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