Survey of microbes in industrial-scale second-generation bioethanol production for better process knowledge and operation

Velma T. E. Aho[1,2] (https://orcid.org/ORCID: 0000-0003-2916-7018),
Tiina Tolonen[3] (https://orcid.org/ORCID: 0000-0001-9426-8662),
Jasmiina Haverinen[3] (https://orcid.org/ORCID: 0000-0001-7414-622X),
Mari Jaakkola[3] (https://orcid.org/ORCID: 0000-0002-6419-1489),
Lars Paulin[1] (https://orcid.org/ORCID: 0000-0003-0923-1254),
Petri Auvinen[1] (https://orcid.org/ORCID: 0000-0002-3947-4778)
and M. Minna Laine[4*] minna.laine@st1.fi, https://orcid.org/0000-0002-5534-4500

[1]Institute of Biotechnology
University of Helsinki
Helsinki, Finland

[2]Department of Neurology
Helsinki University Hospital
Helsinki, Finland

[3]Unit of Measurement Technology, Kajaani University Consortium
University of Oulu
Kajaani, Finland

[4]St1 Oy
Helsinki, Finland

Abstract

The microbes present in bioethanol production processes have been previously studied in laboratory-scale experiments, but there is a lack of information on full-scale industrial processes. In this study, the microbial communities of three industrial bioethanol production processes were characterized using several methods. The samples originated from second-generation bioethanol plants that produce fuel ethanol from biowaste, food industry side streams, or sawdust. Amplicon sequencing targeting bacteria, archaea, and fungi was used to explore the microbes present in

biofuel production and anaerobic digestion of wastewater and sludge. Biofilm-forming lactic acid bacteria and wild yeasts were identified in fermentation samples of a full-scale plant that uses biowaste as feedstock. During the 20-month monitoring period, the anaerobic digester adapted to the bioethanol process waste with a shift in methanogen profile indicating acclimatization to high concentrations of ammonia. Amplicon sequencing does not specifically target living microbes. The same is true for indirect parameters, such as low pH, metabolites, or genes of lactic acid bacteria. Since rapid identification of living microbes would be indispensable for process management, a commercial method was tested that detects them by measuring the rRNA of selected microbial groups. Small-scale testing indicated that the method gives results comparable ~~to~~with plate counts and microscopic counting, especially for bacterial quantification. The applicability of the method was verified in an industrial bioethanol plant, inspecting the clean-in-place process quality and detecting viability during yeast separation. The results supported it as a fast and promising tool for monitoring microbes throughout industrial bioethanol processes.

Keywords
Process hygiene
Bioethanol
Amplicon sequencing
Lactobacilli
rRNA sandwich hybridization

1. Introduction

Biofuels, for example, bioethanol, could be of key importance in lowering carbon dioxide emissions to combat global warming (Oh et al. 2018). Currently, bioethanol is mainly produced from biomass related to edible materials, such as sugarcane or molasses in Brazil or corn in the U~~nited~~ SA~~tates~~. Second-~~-~~generation bioethanol based on waste streams would be a better, more sustainable alternative.

Good process hygiene is vital for running a bioethanol plant in a qualitative, economic, and efficient way. Microbes that invade industrial processes may reduce ethanol yield and/or spoil fodder. Lactic acid bacteria have been identified as main spoiling organisms in industrial

bioethanol processes using sugarcane or molasses (Brexó and Sant'Ana 2017; Costa et al. 2015; Dellias et al. 2018; Lucena et al. 2010), as well as in corn ethanol plants (Li et al. 2016; Rich et al. 2018; Skinner and Leathers 2004), and means to limit their occurrence have been suggested (Albers et al. 2011; Beckner et al. 2011; Ceccato-Antonini 2018; Costa et al. 2018; Leathers et al. 2014; Leite et al. 2013; Muthaiyan et al. 2011; Saunders et al. 2019; Shaw et al. 2016). Less is known about the spoiling microbes in bioethanol production processes using other feedstocks. Identification of the microbial species involved gives information about their properties and is thus useful when choosing the correct actions for removing them. In Europe, the use of antibiotics, the most common approach in bioethanol processes elsewhere in the world, is generally not accepted. As a consequence, other means to control spoilage causing microbes are necessary.

When a fuel ethanol plant is operated as a biorefinery, there are other microbial communities to be considered in addition to those related to ethanol production itself; successful wastewater treatment is also an important biological and environmental aspect of the process. Several studies have already described the microbial distribution and acclimatization, especially of archaea, in full-scale anaerobic digesters (Cardinali-Rezende et al. 2012; Ince et al. 2010; Leclerc et al. 2004; Lee et al. 2014; Li et al. 2018; Röske et al. 2014; Sundberg et al. 2013; Yan et al. 2019).

Many ethanol-producing processes take place in anaerobic or microaerophilic conditions, making normal plate counts for total aerobic bacteria not applicable when following microbial contamination of those process steps. Additionally, some microbes, such as methanogenic archaea, which are the main players in methane production in biogas reactors (Yu et al. 2010), are non-culturable. This means that instead of plate count techniques, molecular detection methods must be used to identify them in order to obtain more information about the microbial status of the biological wastewater treatment step.

If known microbes, for example, lactic acid- or sulphfate-reducing bacteria, are constantly causing problems in a bioethanol production process, a fast monitoring method such as quantitative PCR can be used to detect and quantify them (Lucena et al. 2010; Moestedt et al. 2013; Muthaiyan and Ricke 2010). DNA-based identification methods, however, detect both living and dead cells. Since living organisms are the ones causing problems in processes, and only living microbes can ferment, the microbial detection method should reveal only active, living cells. Furthermore, when managing actual full-scale processes, the identification method

needs to be fast, inexpensive, and applicable on industrial scale.

The Finnish energy company St1 is a pioneer in waste-based ethanol production and related technologies. Since 2016, St1 Oy has operated a demonstration-scale Cellunolix® bioethanol plant that uses sawdust as feedstock and has an annual production capacity of 10 million liters of fuel ethanol. The company also owns and operates several smaller biorefineries that utilize biowaste (Bionolix®) or bakery and grocery waste (Etanolix®) as feedstock, and produce heat and electricity, animal feed, and/or soil amendments together with fuel ethanol.

The bioethanol production process in a St1 Oy biorefinery starts with pretreatment of raw material, either biochemically or physicochemically, depending on the material. Partly dissolved cellulose and starch are further hydrolyzed enzymatically, and the clear liquid containing monosaccharides is separated for yeast fermentation. The dilute ethanol formed during the fermentation is distilled, and the side streams, including vinasse and wastewater, are further processed. Wastewater is biologically treated either in a continuous stirred-tank reactor (CSTR) or in an internal circulation (IC) reactor, followed by methane production. Process water is partly recirculated. The IC reactor is an anaerobic digester developed from the upflow anaerobic sludge blanket (UASB) reactor (Driessen et al. 1999).

Since St1 Oy's industrial-scale bioethanol plants are unique, there is a negligible amount of published information relevant to their microbial populations, function, or operability. The overall goal of this study was a better understanding of these specific processes, which would also provide more information on industrial-scale operation of bioethanol plants in general. The workflow consisted of three steps. The first step was to screen the microbial communities in the processes using amplicon sequencing, terminal restriction fragment length polymorphism (TRFLP), and traditional plate counts to (a) identify spoiling micro-organisms possibly present in fermentation samples from Etanolix® and Bionolix® plants, and to (b) see how well anaerobic digestion process is adapted to the quite unique influent, which was biowaste-based stream after ethanol fermentation. In the Bionolix® process, CSTR that is used to treat wastewater and sludge cannot be operated by changing feeding material. This sets it apart from processes such as agricultural reactors, where for example cattle manure can be used to stabilize reactor performance and to equilibrate C/N ratio in the feedstock. In Bionolix®, the stream is always the same, and due to the closed process, no additional streams are allowed.

The microbial community screening produced useful information, for example revealing the presence of biofilm bacteria in the fermentation process, but also made it clear that indirect methods (genes or metabolic products of spoiling bacteria such as lactic acid) did not indicate the presence of active microbes, so another approach was needed for the detection of living organisms. Finding a suitable method was the motivation for the second step of the study, which was performed with a new set of fresh samples. The specific method tested was a sandwich hybridization kit (HybriScan), originally developed for breweries, which is based on measuring rRNA of selected microbial groups. The results were compared ~~to~~with more traditional methods such as plate counts and hemocytometric measurements. In the third step, new methods that had been tested in smaller scale in the second step were taken into real-life situations in the industrial scale at Cellunolix® plant, to monitor clean-in-place (CIP) protocol and to study yeast viability during separation after fermentation.

2. Materials and methods

2.1. Samples

Samples were collected from the St1's full-scale bioethanol plants and smaller-scale test reactors. To characterize the microbial communities present in different process steps, a total of 46 samples were collected from Etanolix® and Bionolix® plants, as well as from full- and laboratory-scale continuous stirred-tank reactors (CSTR) using Bionolix® plant feed, and pilot-scale internal circulation (IC) reactors with simulated Cellunolix® wastewater. These samples were analyzed using amplicon sequencing, TRFLP, or both (Table 1; Supplementary Table S1). Most of the samples were frozen before sequencing, but a subset (*n* = 22) w~~ere~~as processed fresh and plate-counted within three days after sampling (Table S1).

To evaluate a commercial rRNA detection kit (HybriScan), a new set of samples was collected. Full-scale Etanolix® (*n* = 9) or Bionolix® (*n* = 3) samples as well as samples from laboratory fermentation tests (*n* = 6) were investigated (Table 2~~1~~; Supplementary Table S1). All of these samples except one were also either plate-counted on corresponding media or the cells were counted using a hemocytometer.

To test this rRNA detection method in an industrial production context, two full-scale events, yeast separator commissioning, and CIP quality check-up~~,~~ were examined at the Cellunolix® plant. The amount of yeast was followed in six samples and the amount of total bacteria in 15 samples (Table ~~1~~3; Supplementary Table S1).

The categorization of fermentation samples as "good," "bad," and "average" performance was based on several factors followed in the industrial-scale operation by monitoring measurements (metadata not shown) and assessments made weekly by process operators and process engineers. Different sampling points during the bioethanol production process had to meet different, specific criteria. For example, a sample moving from hydrolysis to fermentation had to have high enough dry matter content and pH within a certain range. After the fermentation, dry matter content had to decrease, pH had to stay at a certain level, and organic acids content had to be low. The animal feed needed to meet certain criteria on dry matter content, low pH, and good hygiene. Some values were followed in everyday operation, and in some cases, more extensive sampling and analysis repertory was used for a set of samples, for example, after more rigorous CIP.

2.2. CSTR monitoring measurements

Dissolved chemical oxygen demand (DCOD) and ammonium nitrogen were analyzed using Hach Lange kits (Hach Lange GmbH). The volatile solids (VS%), total solids (TS%), volatile fatty acids (VFA), and alkalinity were measured as described by Ritari et al. (2012). Gas composition from the biogas reactor was analyzed using a GA5000 portable gas meter (Geotechnical Instruments Ltd). These measurements are part of the weekly operational monitoring of the Bionolix® CSTR.

2.3. Amplicon sequencing

To screen microbial communities using amplicon sequencing (Gołębiewski and Tretyn 2020), bulk DNA was extracted from the samples with the FastDNA SPIN Kit for Soil (MP Biomedicals), and the target region V1–V3 (bacteria) or V3–V4 (archaea) of the 16S rRNA gene, or ITS2 (fungi) was amplified with PCR. For bacteria, the amplification followed a previously published protocol (Salava et al. 2017); a similar protocol, but with different primers (Supplementary Table S2), was used for archaea and fungi. The amplicons were sequenced on the Illumina MiSeq platform (paired-end, with 326 bp for the forward reads and 286 bp for the reverse reads).

Sequences were curated using mothur (version 1.35.0), following the Standard Operating Procedure for MiSeq data (Kozich et al. 2013; Schloss 2015; Schloss et al. 2009) for archaea and bacteria, and an adjusted workflow that omits the "align.seqs" and "pre.cluster" step and uses

"pairwise.seqs" instead of "dist.seqs" for fungi. The taxonomy references used were the RDP 16S rRNA reference (PDS, version 10) for archaea and bacteria, and the UNITE database (version 6) (Kõljalg et al. 2013) with a manually added set of plant ITS sequences for fungi. Sequences were further tentatively annotated to the species level using EMBL-EBI's BLAST+ search, with the NCBI non-redundant nucleotide sequence collection as the reference database. The results are presented as relative abundances of operational taxonomic units (OTUs) in each sample.

The raw amplicon sequence data has been uploaded to the European Nucleotide Archive (accession number PRJEB31049), including several additional process samples not discussed in this study.

2.4. Bacterial community profiling

Bacterial community profiles of samples were resolved using a TRFLP (De Vrieze et al. 2018) analysis protocol modified from Phusion Bacterial Profiling Kit (Thermo Scientific). Approximately 200 µL of filtered sample was used for the extraction of bacterial DNA with PrecipitorR 16 (Abnova). Extracted DNA was amplified with PCR using universal 16S ribosomal RNA primers 8F and 926R for bacteria labelled with 6-FAM and NED fluorescence dyes respectively, and PCR product purified with Nucleospin Gel and PCR Clean-up kit (Macherey Nagel). Cleaned PCR product was digested with restriction enzymes MSpI and Hin6I, and the resulting fragments were analyzed in Biocenter Oulu Sequencing Center (University of Oulu) using capillary electrophoresis. The detected fragments were compared with a modified Phusion Bacterial Profiling Kit database (Thermo Scientific) to define the most abundant bacterial genera in the sample.

2.5. 2.6.2.5. Lactic acid

Prior to analyses, samples were pre-filtered with a 225-µm filter paper. Filtered samples were centrifuged (16,800-*g*/10 min), and supernatants were filtrated with 0.45-µm GHP Acrodisc syringe filters and diluted with water. Quantitative analysis of lactic acid was performed with a P/ACE MDQ CE instrument (Beckman-Coulter) equipped with a diode array detector (DAD) according to a previously published method (Schneider et al. 2016). Quantification was based on the calibration curve of the standard solution of lactic acid. The limit of detection and limit of quantification (calculated according to Keith et al. 1983) for lactic acid in CE analysis were 1.9 mg/L and 5.1 mg/L, respectively.

~~2.7.~~2.6. Cell counts

Viable yeast cells were quantified based on methylene-blue-~~-~~stained cells using a Neubauer-improved counting chamber (Bastidas 2013). Microbial plate counts for total bacteria, yeast, mold, and fungi were determined using standard techniques and cultivation media (SFS 1999; NMKL 2005; NMKL 2013). Lactic acid bacteria were enumerated using standard procedure but modified temperature of 30 ~~-~~°C instead of 22 ~~-~~°C (NMKL 2007).

~~2.8.~~2.7. Detection based on rRNA

The HybriScan™ (ScanBec GmbH) method detects ribosomal RNA of groups of microorganisms, such as total bacteria, lactic acid bacteria, or spoiling yeasts, using colorimetric sandwich hybridization on microtiter plates (Bamforth 2016; Huhtamella et al. 2007; Rautio et al. 2003; Siegrist et al. 2015). The method was developed for the beer, food, and beverage industries. The HybriScan D Lactobac kit measures 27 different spoiling bacteria from genera *Lactobacillus*, *Pediococcus*, *Pectinatus*, and *Megasphaera*, while the HybriScan D Total Bacterial Count kit targets the eubacteria. The HybriScan D Yeast kit detects almost 30 species from genera *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Candida*, *Torulaspora*, *Brettanomyces*, *Debaryomyces*, and *Hanseniaspora* (Sigma-Aldrich 2019). Hence, HybriScan™ kits detect the same microbial groups that are relevant in the fuel ethanol industry. The enumeration of microbes was performed according to each kit's instructions. Samples were diluted in broth, but not pre-grown, to enable semi-quantitative analysis. Fresh samples were diluted and measured in two replicates of two different dilutions.

3. Results

3.1. Microbial community screening: Etanolix® and Bionolix® bioethanol production processes

To gather more information and to get an overview of the microbes in the different process steps in bioethanol plants, bacteria and fungi present in Etanolix® and Bionolix® samples were screened using amplicon sequencing, TRFLP, and lactic acid measurements (Table ~~1~~2, Supplementary Table S1). These samples represented various process steps: raw material, hydrolysis, and fermentation, and for Etanolix®, also quality control for the animal feed side product.

In the amplicon-~~-~~sequenced samples from the full-scale ethanol production plant, Etanolix®,

Saccharomyces cerevisiae was the main identified fungal species in samples taken on good or average days. When the ethanol production was poor, the community also included additional yeast species, the most abundant of which were *Candida humilis*, *Pichia burtonii*, *Alternaria metachromatica*, and *Saccharomycopsis fibuligera*, mainly in the hydrolysis step (Fig. 1a). For bacteria, most of the identified OTUs in Etanolix® samples represented lactobacilli or acetic acid bacteria (Fig. 1b). The raw material for the Etanolix® plant varied, being either bread or dough, which most likely reflected in the different microbial communities seen in the corresponding samples. The relationship between good operation and abundances of certain bacterial species was not as clear as for the fungal analyses, but ethanol production was typically good in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the fermentation (Fig. 1b).

In the Bionolix® plant, which uses grocery and household biowaste, the raw material varied extensively, as did the bacterial profile in the hydrolysis step (Fig. 2). The raw material was heated in the beginning of the hydrolysis step for hygienization. Regardless of the different bacteria present in incoming biowaste, the bacterial profile in fermentation was distinctly similar (Fig. 2). The most prominent bacterial species in fermentation were *Lactobacillus amylovorus* and *L. pontis*, followed by *L. fermentum*, *L. panis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *L. amylolyticus*. Four Bionolix® fermentation samples were included in the amplicon sequencing dataset targeting fungal taxa. The relative abundances of *Saccharomyces cerevisiae* and *Candida humilis* in these four samples were 50:50, 81:19, 42:58, and 30:70 %, respectively. This indicated high relative abundance of spoiling yeast, ranging from 19 to 70% (average 49%), but also high variability between the samples.

In addition to amplicon sequencing and TRFLP, which detect microbes directly based on genetic material, the presence of lactic acid bacteria in fermentation samples can be estimated indirectly by measuring the lactic acid concentration. The lactic acid in the Etanolix® process samples was formed during the fermentation step as a metabolite of lactic acid bacteria. In the Bionolix® samples, lactobacilli and lactic acid were found in higher numbers already in the raw material (first hydrolysis sample), indicating that the lactic acid originated from partly decomposed biowaste (Table ~~2~~4). Based on the plate counts, a heating step, either for hygienization or as a consequence of distillation or evaporation, decreased the amount of vegetative lactic acid bacterial cells from the next process step (Table ~~2~~4). The other measurements, such as prominence of lactic acid bacteria in TRFLP profiles, low pH, or concentration of lactic acid in process samples, did not correlate as clearly with the amount of living lactobacilli. Lactic acid,

especially when produced in high quantities during fermentation, remained in the liquid, even though cells were heat-inactivated (Table ~~2~~4). Thus, the presence of certain genes or metabolic products did not indicate that the cells were growing or otherwise active at that point.

3.2. Microbial community screening: anaerobic digestion of bioethanol plant wastewater and sludge

To learn more about the fitness of the wastewater and sludge treatment units in St1 biorefineries, archaea and bacteria were amplicon-~~-~~sequenced from the full-scale CSTR reactors managing wastewater from the Bionolix® process, and from laboratory CSTR and IC reactors handling Bionolix®- and Cellunolix®-simulated wastewater (Table 1; Supplementary Table S1).

The seed (granular sludge) for the IC reactor laboratory testing originated from the forest industry. A second granular sludge sample was taken in the middle of a test when the granule died, and a third and final granule sample after a successful reactor operation. Based on amplicon sequencing results, the main species in all three IC reactor samples was *Methanosaeta concilii*, of the order *Methanosarcinales* (Fig. 3). The microbial community at the start of the IC reactor test was very similar to the one that did not survive. The one that reached steady-state operation with Cellunolix®-type wastewater evolved so that the proportion of thermophilic archaea decreased and the proportion of order *Methanobacteriales* increased (Fig. 3). The main genus was *Methanobacterium*. A sequence similarity search using BLAST+ gave the species *Methanobacterium subterraneum* as the closest hit to the most abundant OTU sequence.

Similarly to the IC reactor, in the beginning of the full-scale Bionolix® CSTR start-up, the main methanogens represented the order *Methanosarcinales* (Fig. 3), and the most abundant species identified by nucleotide database similarity search was again *Methanosaeta concilii.* On days 338 and 345, methanogen distribution in the Bionolix® CSTR samples was different (Fig. 3). During those days, the full-scale CSTR was not working optimally, as can be seen from the lower methane production, higher concentration of volatile fatty acids (VFA) and high VFA/alkalinity ratio (over 0.80) (for monitoring results, see Supplementary Table S3). A new species, with a 16S rRNA gene sequence similar to an uncultured *Methanobacterium* sp., was transiently detected during those days.

Methanomicrobiales were the main hydrogenotrophic methanogens found in Bionolix® CSTR samples (Fig. 3), where the genus *Methanoculleus* dominated and its proportion increased over

time. The representative sequence of the most abundant *Methanoculleus* OTU resembled that of the species *Methanoculleus bourgensis*. The relative abundance of the order *Methanobacteriales* decreased during operation; this order mainly consisted of sequences representing the genus *Methanobacterium*. The methanogen distribution approached that of the CSTR profile reported by Lee et al. 2014 (Fig. 3). After running for almost ~~two~~ 2 years, the Bionolix® CSTR had a diverse bacterial population, where many sequences were similar to non-culturable organisms isolated from laboratory- or full-scale biogas reactors (Table ~~3~~5).

3.3. Small-scale testing of the rRNA detection kit: living micro-organisms in bioethanol process samples

Based on the initial screening of microbial groups in Etanolix® and Bionolix® samples, it was clear that indirect methods (genes or metabolic products of spoiling bacteria such as lactic acid) did not indicate the presence of active microbes, and that additional methods should be explored for the detection of living organisms. As a possible method for achieving this goal, rRNA detection kits that detect group-specific rRNA strings by sandwich hybridization were tested with Etanolix® and Bionolix® samples and laboratory fermentation test samples.

First, fresh samples were collected from different steps of Bionolix® and Etanolix® bioethanol production. The results from these kits detecting total and lactic acid bacteria were compared with that of~~to~~ plate counts. Traditional plate counts and the rRNA detection measurements gave comparable results for the amount of total bacteria. The correlation between lactic acid bacteria rRNA and plate counts was, on the other hand, not as evident (Supplementary Fig. S1).

The rRNA detection kit for spoiling yeast is designed to detect rRNA from a wide variety of spoiling yeast species in beer-making. Here the kit was tested to follow viability of the process yeast *Saccharomyces cerevisiae*. To test the rRNA detection kit for yeast, six different laboratory fermentation samples were analyzed first (Table ~~1~~2, Supplementary Table S1). From the same samples, yeast cells were quantified with a hemocytometer and/or plate counts (Table ~~4~~6). Correlation between the different methods was most evident in the samples taken in the early phase of fermentation, where the yeast cells were freshly added (Table ~~4~~6).

3.4. Full-scale testing of the rRNA detection kit: clean-in-place performance and yeast viability during separation

The validity of using the rRNA detection method in an industrial setting was explored in full-

scale operation of the Cellunolix® plant in two cases: verifying clean-in-place (CIP) performance, and monitoring yeast viability during yeast separation after fermentation. The results from the rRNA detection kit for total bacteria were again compared ~~to~~with heterotrophic plate counts, in order to determine the success of CIP~~-~~ protocol in the Cellunolix® hydrolysis and fermentation tanks. Five fermentation tanks and four hydrolysis tanks were sampled before and after the clean-in-place step, and rRNA for total bacteria was measured from 15 samples (Table 7~~5~~). Given the inaccuracy of heterotrophic plate counts caused by only one plated dilution, the results were in good accordance with the results of the rRNA detection kit for total bacteria (Table ~~5~~7).

During the full-scale commissioning of the yeast separator, different separation parameters were tested, and the separated streams were sampled. On three separation occasions, the amount of living yeast was quantified using the rRNA detection kit for yeast. The fermentation tanks were let to stay for a prolonged time up to 240 ~~-hours~~ to have several separation events in a row. Since the fermentation had been completed several days before separation tests, most of the yeast cells were in the death phase during the separation test. Therefore, the amount of detectable yeast cells with the rRNA-based kit was low (Table 8~~6~~).

4. Discussion

4.1. Amplicon sequencing reveals spoiling microbes in bioethanol production processes

Many biofilm bacteria were identified in both the Etanolix® and Bionolix® processes through amplicon sequencing. In spite of the large variation in microbial species within hydrolysis samples, the fermentation samples from Bionolix® had noticeably similar profiles including *Lactobacillus amylovorus*, *L. pontis*, and *L. amylolyticus*. The two first-mentioned lactic acid bacteria have been previously detected from a biofilm in the fuel ethanol process (Skinner-Nemec et al. 2007). The result suggests that there was a biofilm contamination between the hydrolysis and fermentation tanks. Here, the identity of the bacterial species and their habitat led to this conclusion and helped to locate the contamination.

Having metadata from the industrial process supported the results and enabled linking samples to "good" or "bad" process days. On a "good" process day, the most abundant bacterial species was *Lactobacillus delbrueckii* subsp. *bulgaricus*, which has been reported to have synergy with *Saccharomyces cerevisiae*. According to chemostat studies by Mendes et al. (2013), *L. delbrueckii* sb. *bulgaricus* transforms lactose, which *S. cerevisiae* cannot use, into glucose and

galactose. *Saccharomyces*, in turn, produces alanine for *Lactobacillus*' usage. Moreover, *L. delbrueckii* sb. *bulgaricus* can only grow in the presence of high carbon dioxide, which the yeast is producing together with ethanol. Additionally, certain *Lactobacillus* species, for example, *L. plantarum*, *L. casei, and L. pontis*, provide an advantage for the fermenting yeast by competing with other lactic acid bacteria, such as *L. fermentum*, whose presence during the fermentation can be more harmful (Rich et al. 2018). Thus, not all of the identified microbes are harmful to fuel ethanol production; on the contrary, some may have a positive effect or even synergy with the fermenting yeast.

Spoiling wild yeasts and fungi, such as *Candida humilis*, *Hyphopichia (Pichia) burtonii*, and *Alternaria metachromatica*, were detected in Etanolix® samples on days when process parameters indicated poor fermentation, while on a good performance day, *Saccharomyces cerevisiae* was the most prominent yeast species. On the other hand, *Saccharomycopsis fibuligera*, which was also detected in Etanolix® samples, can have a positive effect on fermentation, because it produces amylolytic and cellulolytic enzymes (Lee et al. 2017). Etanolix® plants use bakery, grocery, and food industry waste, such as excess bread, dough, and biowaste as feedstock. Among the most abundant fungi in sourdough are *Saccharomyces cerevisiae*, *Candida humilis*, *Kazachstania exigua*, *Pichia kudriavzevii*, *Wickerhamomyces anomalus*, and *Torulaspora delbrueckii* (De Vuyst et al. 2016). *Candida humilis*, in particular, is a common organism in the rye sourdough fermentation process (Brandt et al. 2004). The origin of these yeasts in Etanolix® samples can also be moldy bread that is present in the grocery waste--based feedstock, since *Pichia anomala*, *Hyphopichia burtonii*, and *Saccharomycopsis fibuligera* are reported to cause chalk mold in for example par-baked bread (Deschuyffeleer et al. 2011).

The Bionolix® fermentation process contained a considerable amount of the wild yeast *Candida humilis*. In some of the fermentation samples, *Candida humilis* was as abundant as *Saccharomyces cerevisiae*. Even though Etanolix® hydrolysis samples had more diverse yeast community, *Saccharomyces cerevisiae* was clearly the most abundant species even on bad operational days. This could be due to the fact that the hygienization step for biowaste is not as complete as in bread and dough waste, so more wild yeasts survive in a process using biowaste as feedstock. After the study presented here, a new clean-in-place protocol has been added to Bionolix® fermentation tanks, and the process hygiene and ethanol yield have considerably improved.

Once the spoiling microbes have been identified, proper tailor-made tools can be used to eliminate them. In Europe, the use of antibiotics in industrial bioethanol production is discouraged. In the case of lactic acid and other gram-positive bacteria, natural antibacterial agents such as hop acids can be used (Leite et.al 2013). If acetic acid bacteria or some *Brettanomyces* yeasts are spoiling fermentation, their number can be decreased by controlling the amount of air in the process, or by adding sulfur dioxide (du Toit et al. 2005). Several lactic acid bacterial species form biofilm, which can be removed with thorough mechanical washing and steam treatment of the reactors and pipelines. Enzymes or lipopeptides produced by *Bacillus* spp. can also prevent biofilm formation (Leathers et al. 2014; Saunders et al. 2019). The most challenging contamination for the fermentation is wild yeast. Sometimes, this challenge can be overcome only by changing the whole fermentation volume (de Souza Liberal et al. 2007; Muthaiyan et al. 2011).

Mixed contamination is very challenging to handle, at least merely by altering environmental conditions (e.g., pH, temperature, biocide addition). Lactic acid bacteria have a very broad pH spectrum: *L. pontis* and *L. panis* can survive even at pH 2.5 for a short period of time (Pedersen et al. 2004). Some lactic acid bacteria are thermophilic, so their optimum temperature is between 37 and 45 °C. Salt (4–6 % NaCl) inhibits the growth of obligately heterofermentative lactobacilli species, but has no effect on wild yeast such as *Candida humilis* (Brandt et al. 2004). And even if the fermentation conditions are adjusted so that most of the wild yeast or lactic acid bacteria cannot thrive, there are extremophiles that can survive under given circumstances, such as *Debaryomyces hansenii* (Prakash et al. 2011), which is an osmo-, halo-, and xerotolerant yeast found in cheese (Breuer and Harms 2006) and sausages (Murgia et al. 2019), possible components in Etanolix® or Bionolix® raw material.

In conclusion, amplicon sequencing gave detailed information about the microbes present in bioethanol processes, and helped in determining the type and location of contamination in the process. For example, biofilm bacteria found in the Bionolix® process pinpointed the contamination within the process. Based on the results, a new CIP protocol has been initialized and the process hygiene has improved. It was also noted that not all identified bacteria in the Etanolix® process were detrimental. Some of them like certain *Lactobacilli* may protect the fermenting yeast from even more harmful spoiling lactic acid bacterial species; others, like *Saccharomycopsis fibuligera*, may help in the fermentation process by excreting hydrolytic

enzymes. Thus, the goal in waste-based bioethanol production is not to destroy all organisms except fermenting yeast, but to have knowledge and to sustain the "controlled chaos" of microbial community balance.

4.2. Microbial community succession in biogas reactors

The most common methanogen in the amplicon sequencing-based microbial community screening of IC test reactor samples belonged to the order *Methanosarcinales*, with a 100% sequence similarity to *Methanosaeta concilii*. *Methanosaeta* are important for granule formation, because they form filaments (Yu et al. 2010). *Methanosaeta concilii* and *Methanosarcina mazei* have been linked to active cells in UASB granular sludge (Díaz et al. 2006). *Methanosaeta* are acetoclastic methanogens of the order *Methanosarcinales* that convert acetate to methane, have fairly long generation times of 3.5 to 9 days, and favor conditions with low acetate level. The order also includes genus *Methanosarcina*, with species that are both acetoclastic and hydrogenotrophic methanogens occurring in higher acetate concentrations, and have a shorter generation time, only 24 hours. Other identified methanogens besides acetoclastic *Methanosaeta* and *Methanosarcina* are hydrogenotrophic methanogens that use H_2 to reduce CO_2 to methane, or to convert formate, methanol, methylamines, or methyl sulfides to methane (Yu et al. 2010).

When the Cellunolix® (lignocellulosic ethanol plant)-simulated wastewater was successfully purified in the IC test reactor, thermophilic methanogens disappeared, and a new species of order *Methanobacteriales* appeared. Based on comparisons against the NCBI nucleotide database, the representative sequence of the most abundant OTU was identical to that of *Methanobacterium subterraneum*. The thermophilic methanogens present in the initial IC reactor granule were identified as order *Thermogymnomonas* and *Thermofilum*. The latter respires S and produces H_2S (Yu et al. 2010):

$2[H] + S^0 \rightarrow H_2S$

The presence of *Thermofilum* may be due to the fact that the granular biomass originated from an IC reactor treating paper mill effluent, where there are sulphfur compounds present.

It is challenging to find relevant published data to compare archaea profiles in full-scale digesters with the CSTR in this study. The results from Lee et al. (2014) were from mesophilic full-scale CSTR and UASB reactors treating wastewater from food waste-recycling. The CSTR results in Röske et al. (2014) were from a 5-L liter test reactor digesting simulated thin stillage from a bioethanol plant. Ince et al. (2010) reported on methanogenic and other archaea from an

industrial CSTR using lees and vinasses as feedstock. Li et al. (2018) studied the effects of increasing organic loading rate in a laboratory-scale CSTR fed with food waste and waste-activated sludge.

During several months of evolution, the distribution of methanogens in the CSTR changed towards a higher abundance of order *Methanomicrobiales*. After more than 600 days' adaptation to Bionolix® bioethanol plant feed, methanogenic groups in the full-scale CSTR shifted from genus *Methanosaeta* in order *Methanosarcinales* towards *Methanoculleus* (*Methanoculleus bourgensis*) in order *Methanomicrobiales*.

Lee et al. (2014) explained the shift in mesophilic CSTR from *Methanosaeta* to *Methanoculleus* with elevated acetate concentration from 1 g/L to almost 2 g/L. Furthermore, *Methanosaeta* species are reported to be sensitive to high ammonium concentration and pH fluctuation (Klang et al. 2015). *Methanoculleus*, in turn, ~~are~~ is mentioned to be robust methanogens. *Methanoculleus* and *Methanosarcina* are found in stable operation of CSTRs (Schmidt et al. 2014; Ziganshin et al. 2016). The abundance of *Methanoculleus* increased in a full-scale farm CSTR which was not inoculated with mature reactor content, but started with stale and cold cattle slurry (Goux et al. 2016).

In the Bionolix® CSTR, the relative abundance of order *Methanosarcinales* decreased over time. Hence, the methanogenic population changed from acetoclastic to hydrogenotrophic archaea. High NH_4^+-N concentration has a negative effect on the acetoclastic methanogenesis pathway. At high ammonia (2.8 g NH_4-N/L) levels, syntrophic acetate oxidation followed by hydrogenotrophic methanogenesis metabolic pathway was found to be dominant, mediated by *Methanobacteriales* spp. and *Methanomicrobiales* spp. (Fotidis et al. 2014). In the CSTR in the present study, ammonium levels were between 2.4 and 3.3 g NH_4^+-N/L. In the laboratory IC tests, ammonium nitrogen concentration never exceeded 0.5 g NH_4^+-N/L. There are, however, almost equally high levels up to 2.3 g NH_4^+-N/L of ammonium nitrogen in a full-scale Cellunolix® IC reactor as in CSTR influent.

Monitoring of archaea during the evolution of a full-scale CSTR consortium showed changes in methanogen groups from acetoclastic to hydrogenotrophic archaea and gave information on adaptation mechanisms for example to high ammonium levels in bioethanol plant feed. A decrease in the abundance of the genus *Methanosarcina* further indicated that the bioethanol

process wastewaters and sludge are not easy substrates for anaerobic digestion. Especially, wastewater from the lignocellulosic ethanol plant mainly consists of organic acids and small amounts of ethanol, as well as alkaline water used for the reactor washing. Furthermore, the wastewater is low in nutrients and trace elements, lacks carbohydrates, is acidic, and contains several inhibitory compounds that are either formed ~~either~~ during the ethanol production process or originate from the lignocellulosic raw material.

Optimal growth conditions for *Methanoculleus bourgensis*, the most abundant methanogen in the full-scale CSTR detected after evolution, are at pH 6.7 and temperature 35–40 °C (Garcia et al. 2006). *Methanobacterium subterraneum*, which became more abundant among methanogen species in a well-working IC test reactor, grows optimally at 20–40 °C, pH 7.8–8.8, and 0.2–1.2 M NaCl (Kotelnikova et al. 1998). For *Methanosaeta concilii*, which was present throughout the test in the IC reactor, the optimal pH is 7.1–7.4 and optimal temperature 35–40 °C (Kendall and Boone 2006). The above-mentioned conditions took place in both mesophilic CSTR and IC reactors in this study.

During the microbial survey reported here, *Methanosaeta concilii* disappeared from the CSTR, but remained in the IC reactor. The reason for this can be that genus *Methanosaeta* uses acetate as a methanogenic substrate, whereas *Methanoculleus* and *Methanobacterium* use formate and H_2 (Liu and Whitman 2008). Maybe the availability of the correct electron acceptor in CSTR feed was not optimal for *Methanosaeta* compared ~~to~~with the IC reactor influent, which is known to contain organic acids, especially acetate. As stated before, also NH_4^+-N levels were lower in the IC test reactors.

Leclerc et al. (2004) studied 44 industrial-, pilot-, or laboratory-scale anaerobic digesters including two full-scale CSTRs, eight full-scale UASB- reactors, and one pilot-scale digester of both types. The archaea distribution was similar to the findings in our study: *Methanosaeta concilii* was the most abundant species, and the families *Methanobacteriales* and *Methanomicrobiales* were well-represented in the samples studied using single-stranded conformation polymorphism (SSCP) analysis. *Methanosaeta concilii* was found in most of the full-scale UASB and CSTR- samples. The next most abundant species in full-scale CSTR samples were *Methanobacterium formicicum* and *Methanosaeta* spp. In UASB reactors, sequences similar to *Methanobacterium subterraneum* were found in several samples, as well as other *Methanobacterium* spp. (Leclerc et al. 2004).

4.3. rRNA-based detection of "good" and "bad" microbes active in a bioethanol production process

DNA detection methods measure both living and dead cells. Indirect methods, such as quantifying metabolites, may give inaccurate results for a specific process phase, for example, if lactic acid is detected in one process step, even though microbes were heat-killed in the previous step. Therefore, a method for specifically measuring living microorganisms was investigated. The method tested here was based on sandwich hybridization of rRNA molecules representing different microbial groups, such as total bacteria, lactic acid bacteria, or yeasts. The method gave comparable results with plate counts of total bacteria, and it was successfully used to monitor a CIP protocol in an industrial bioethanol plant. The rRNA detection method could be used within a working day to follow the success of cleaning hydrolysis and fermentation tanks. In a fully operating bioethanol plant, fast detection methods are needed in order to adjust the industrial process online.

In a bioethanol production process, it is also important to be able to detect and quantify living yeast cells, not just spoiling microbes. The rRNA detection kit for spoiling yeast was tested during the commissioning of a yeast separation system in the Cellunolix® plant. The rRNA hybridization and hemocytometer cell counting results were not entirely consistent. This may be because hemocytometry is based on microscopic image evaluation and counting of cells. Consequently, when cells are distinguished as living (whole), budding, or dead (colored) cells, an overestimation of living cells may occur. The best agreement between the rRNA detection kit for spoiling yeast and another method was for plate counts of fresh yeast suspension (yeast cream), and yeast counts from the early fermentation step. Especially for samples taken from the end of fermentation in laboratory tests and from yeast separation test in full-scale, the rRNA detection kit for spoiling yeast suggested substantially lower amounts of cells than the microscopic counts (data for full-scale test not shown). In these later phase fermentation samples, glucose has been used up, and yeast is starving and decaying, so no growth occurs, and thus, less rRNA is detected. Both the rRNA and hemocytometer detection methods need expertise and practice. Repeated measurements from similar samples, as well as screening for the most suitable dilutions, might add accuracy for enumeration of yeast cells in bioethanol process samples. Nevertheless, the results from the rRNA detection kit for spoiling yeast should be regarded as semi-quantitative, corresponding to the design of the method. Despite its technical limitations, the rRNA detection method proved to be a good general tool for monitoring the

living cells in multiple samples, especially for detecting spoiling bacteria, and when auditing the performance of a CIP protocol in a full-scale bioethanol factory.

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Compliance with Ethical Standards

Conflict of Interest

Velma T.E. Aho declares that she has no conflict of interest. Tiina Tolonen declares that she has no conflict of interest. Jasmiina Haverinen declares that she has no conflict of interest. Mari Jaakkola declares that she has no conflict of interest. Lars Paulin declares that he has no conflict of interest. Petri Auvinen declares that he has no conflict of interest. M. Minna Laine is employed by ST1 Oy. St1 Oy has commercial interest in bioethanol production.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

A. Electronic supplementary material

ESM 1 (PDF 265 kb)

~~This article contains electronic supplementary material.~~

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Fig. 1 Relative abundances of microbial species identified by amplicon sequencing of different steps of the Etanolix® process. Sample codes: raw, = feedstock for fermentation; Hydr, = hydrolysis; Ferm, = fermentation. Sample days were categorized as good, average, or poor operation based on process metadata. **a** Fungal species; **b** Bacterial species.

Fig. 2 Relative abundances of bacterial species identified by amplicon sequencing from the Bionolix® hydrolysis (Hydr) and fermentation (Ferm) steps on four different sampling dates

Fig. 3 Relative abundances of methanogenic and other archaea in a full-scale CSTR connected to the Bionolix® process (days 1 to 672); in a laboratory-scale CSTR; and in granular sludge from a laboratory test with an IC reactor treating wastewater from the Cellunolix® process, shown next to previously published data and grouped according to order. Sample codes: Cellunolix lab start, = granular sludge in the beginning of the laboratory test; Bionolix full, = full-scale CSTR samples; day 1, = in the beginning of the full-scale reactor start-up; Bionolix lab, = laboratory-scale CSTR; Cellunolix lab, = laboratory-scale IC reactor samples; Cellunolix lab not survived, = granular sludge from unsuccessful laboratory test, where the reactor microbes did not survive; and Cellunolix lab survived, = granular sludge from the successful laboratory test

Table 1 Analysis methods, processes, and process stages sampled in this study. Samples were collected over a time span of 4.5 years; ~~, with~~ the samples are ~~in subsection A~~ collected first ~~and the samples in C last~~. ~~In A,~~ Process stages with multiple samples represent different days of the continuously running industrial production process. For detailed information about the samples, see Supplementary Table S1. Microbial community screening, days 261–1028 (*n* = 46)

Sample origin	Process stage	Amplicon sequencing (*n* = 17)	T-RFLP (*n* = 17)	Both methods (*n* = 12)	Microbial enumeration
Etanolix® (*n* = 25)	Raw material	2	2	0	Plate counts (*n* = 15)
	Hydrolysis	3	4	2	
	Fermentation	3	4	2	
	Animal feed (side product)	0	2	1	
Bionolix®	Hydrolysis	0	2	3	Plate counts

(*n* = 18)	Fermentation	0	1	4	(*n* = 7)
	Anaerobic reactor influent	0	2	0	
	Anaerobic reactor effluent	5	0	0	
	Anaerobic bioreactor laboratory test	1	0	0	
Laboratory (*n* = 3)	UASB granular sludge (test reactor, simulated Cellunolix® wastewater)	3	0	0	

Table 2B. Analysis methods, processes, and process stages sampled in this study. Samples were collected over a time span of 4.5 years. Small-scale testing of rRNA detection kits, days 1331–1459 (*n* = 18)

Sample origin	Process stage	*n*	rRNA group detected	Microbial enumeration
Bionolix® (*n* = 3)	Hydrolysis	1	Total bacteria, yeasts	Plate counts (all samples)
	Fermentation	2	Total bacteria, lactic acid bacteria, yeasts	
Etanolix® (*n* = 9)	Raw material	2	Total bacteria, lactic acid bacteria, yeasts	Plate counts (all samples)
	Hydrolysis	3	Total bacteria, lactic acid bacteria, yeasts	
	Separation after hydrolysis	2	Total bacteria, lactic acid bacteria	
	Fermentation	2	Total bacteria	
Laboratory (*n* = 6)	Fermentation experiment with	6	Yeasts	Plate counts (all samples),

	process yeast			microscopic counts (*n* = 5)

C. Table 3 Analysis methods, processes, and process stages sampled in this study. Samples were collected over a time span of 4.5 years. Full-scale testing of rRNA detection kits, days 1693–1850 (*n* = 21)

Sample origin	Process stage	n	rRNA group detected	Microbial enumeration
Cellunolix® (*n* = 21)	Yeast separator commissioning	6	Yeasts	Microscopic counts (*n* = 5)
	Clean-in-place (CIP) process verification	15	Total bacteria	Plate counts for total aerobic bacteria (all samples)

Table 42 Direct and indirect measurements of the presence of lactic acid bacteria in different process steps of the Bionolix® and Etanolix® processes. Sample codes: Raw = feedstock for fermentation, Hydr = hydrolysis, Fan = solid separation before fermentation, Ferm = fermentation, Dist = distillation. CFU = colony forming unit

	Bionolix®					Etanolix® 1						Etanolix® 2					
	Hydr	Fan	Hydr	Ferm	Dist	Raw	Hydr start	Hydr end	Ferm start	Ferm end	Animal feed	Raw	Hydr start	Hydr end	Ferm start	Ferm end	Animal feed
Heating preceded the sample point	x	x			x						x						x
pH			5.2	3.6		4.6	4.6	4.6	3.7	3.1	3.1	4.7		4.6	3.5	3.2	3.1
Lactic acid concentrat	7.6	2.7	25.6	26.8	42.3	2	1.5	2.3	3.8	9.2	2.1	3.3	2.5	3.1	6.7	13.8	11.4

ion (g/L)																
Lactic acid bacteria plate count (log10 CFU/g)	≤ 3	≤ 3	6.1	8.4	≤ 3	6.2	7.3	7.1	6.8	7.8	≤ 3	5.4	6.4	6.8	9.3	8.1	≤ 3
Lactobacillus identified as main organism by TRFLP	x			x	x						x		x	x			x

Sample codes: *Raw*, = feedstock for fermentation; *Hydr*, = hydrolysis; *Fan*, = solid separation before fermentation, *Ferm*, = fermentation; *Dist*, = distillation; *CFU*, = colony-forming unit

Table 5 Comparison of sequences from the most prevalent OTUs in the Bionolix CSTR sample taken on day 672 and bacterial sequences in the NCBI nucleotide sequence collection. The sequence similarity was 100% unless otherwise noted. The OTUs presented exceeded 100 sequence reads. The search was performed using BLASTN 2.7.1+, on July 8th, 2019

OTU initial identification	Relative abundance, %	Closest match from BLAST search	Isolation source	Reference
Otu008: *Proteiniphilum* (100)	37	EM_ENV:MF769214 Uncultured bacterium clone 40144 16S ribosomal RNA gene, partial sequence	Biogas plant	Klang et al. 2019
Otu065: *Anoxynatronum* (100)	9	EM_ENV:JN998150 Uncultured Firmicutes bacterium clone SAO3 B19	Anaerobic lab-scale digester	Arthurson V., Muller B., Schnurer A.

		16S ribosomal RNA gene, partial sequence		Unpublished.
Otu043: *Kandleria* (100)	5	EM_ENV:MF769136 Uncultured bacterium clone 39832 16S ribosomal RNA gene, partial sequence	Biogas plant	Klang et al. 2019
Otu016: unclassified (100)	5	EM_ENV:LT607884 Uncultured bacterium partial 16S rRNA gene, clone 031-H4-IB29945	Biofilms of a mesophilic methane reactor of a two stage biogas reactor	Bergmann I., Klocke M. Unpublished.
Otu010: *Rikenella* (93)	3	EM_ENV:LN869391 Uncultured Bacteroidetes bacterium partial 16S rRNA gene, isolate OTU 31	Sludge digester	Huber et al. 2016
Otu147: *Saccharofermentans* (100)	3	EM_ENV:JX101985 Uncultured Bacteroidetes bacterium clone BL1_7 16S ribosomal RNA gene, partial sequence. Sequence similarity 99.,4 %	Digest of municipal solid waste full-scale anaerobic reactor	Cardinali-Rezende et al. 2012

Table 6 Detection of *Saccharomyces cerevisiae* in different laboratory fermentation set-ups using plate counts, microscopic cell counting and the rRNA detection kit for spoiling yeast. Estimated amount of yeast was calculated based on the weight of the yeast cream added to each test. YPD = yeast peptone dextrose medium, NA = not analyzed

Sample type	Estimated amount of yeast in sample, cells/ml	Plate counts on YPD, CFU/ml	Microscopic count of living cells, cells/ml (% viability)	rRNA detection kit, CFU/ml, (average ± stdev, (Cv-%)

Separated yeast	3 x 10^8	1.2 x 10^8	2 x 10^8 (98 %)	1.5 ± 0.1 x 10^7 (5 %)
Basic laboratory fermentation	4 x 10^8	7 x 10^8	1.6 x 10^8 (100 %)	2 ± 0.1 x 10^9 (4 %)
Late fermentation	1 x 10^7	< 10^5	2.8 x 10^7 (75 %)	3.6 ± 0.8 x 10^5 (23 %)
Early fermentation, raw material no 1	1 x 10^7	3 x 10^7	5 x 10^7 (97 %)	4.9 ± 0.7 x 10^7 (15 %)
Early fermentation, raw material no 2	7 x 10^6	3 x 10^6	NA	2.8 ± 1.3 x 10^7 (49 %)
Yeast cream (yeast separation / inoculum)	5 x 10^9	4 x 10^9	NA	2.2 ± 0.3 x 10^9 (11 %)

YPD, yeast peptone dextrose medium; *NA*, not analyzed

Table 7 Monitoring the performance of clean-in-place (CIP) protocol in Cellunolix® hydrolysis and fermentation tanks. Plate counts of heterotrophic bacteria were performed according to Finnish standard (SFS-EN ISO 6222 (1999)), except for the cultivation temperature, which was 30 °C. The detection limit depended on the dilution used in plate counts. The rRNA detection range for non-diluted sample was 9 × 10^3 – 5 × 10^5 CFU/mL. ~~< *det.* = under detection. *ND* = not determined~~

Tank type	Before CIP _CFU/mL			After CIP _CFU/mL		
	rRNA detection of total bacteria	Plate counts	pH	rRNA detection of total bacteria	Plate counts	pH
Fermentor 1	3.7 -× -10^5	> -6.5 -× -10^4	8.27	< det.	22	9.58
Fermentor 2	1.4 -× -10^5	1.6 -× -10^6	5.24	< det.	3	9.24
Fermentor 3	ND	ND	ND	< det.	12	9.51
Fermentor 4	ND	ND	ND	< det.	150	9.82
Fermentor 5	1.3 -× -10^5	>- 5 -× -10^3	11.41	< det.	2	13.14
Hydrolysis tank 1	>- 5.2 -× 10^6	8.0 -× -10^7	4.63	ND	ND	ND
Hydrolysis tank 2	2.5 -× -10^6	7.2 -× -10^7	4.35	< det.	4	13.18
Hydrolysis tank 3	2.4 -× -10^6	7.9 -× -10^7	4.42	< det.	1	12.93
Hydrolysis tank 4	2.0 -× -10^5	>- 6.5 -× -10^4	4.94	< det.	2	12.97

< *det*., under detection; *ND*, not determined

Table 8 Full-scale test of yeast separation after fermentation. The amount of yeast was estimated via plant automation from the weight of the yeast cream fed to each of the 600 -- m^3 fermentation tank in the beginning of the fermentation. ~~< det = under detection~~

Yeast separation sample	Estimated yeast amount in the	rRNA detection kit for

	beginning of fermentation, cells/ml	spoiling yeast, CFU/ml average ± stdev (Cv-%)
1st feed to separator (fermentation 119 h)	5 × 10^9	8.4 ± 0.9 × 10^4 (10.6 %)
1st separated yeast		3.2 ± 0.02 × 10^7 (0.7 %)
2nd feed to separator (fermentation 236 h)	4 × 10^9	3.2 ± 0.5 × 10^4 (14.8 %)
2nd separated yeast		< det
3rd feed to separator (fermentation 240 h)	4 × 10^9	3.8 ± 0.6 × 10^4 (16.0 %)
3rd separated yeast		2.8 ± 0.2 × 10^6 (8.0 %)

< *det*, under detection

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Supplementary material

Survey of microbes in industrial scale second generation bioethanol production for better process knowledge and operation

Velma T. E. Aho[1,2] (ORCID: 0000-0003-2916-7018), Tiina Tolonen[3] (ORCID: 0000-0001-9426-8662), Jasmiina Haverinen[3] (ORCID: 0000-0001-7414-622X), Mari Jaakkola[3] (ORCID: 0000-0002-6419-1489), Lars Paulin[1](ORCID: 0000-0003-0923-1254), Petri Auvinen[1] (Orcid:0000-0002-3947-4778) and M. Minna Laine[4]

(1) Institute of Biotechnology, University of Helsinki, Helsinki, Finland
(2) Department of Neurology, Helsinki University Hospital, Helsinki, Finland
(3) Unit of Measurement Technology, Kajaani University Consortium, University of Oulu, Kajaani, Finland
(4) St1 Oy, Purotie 1, P.O.Box 100, FI-00380 Helsinki, Finland. Corresponding author. Email minna.laine@st1.fi, https://orcid.org/0000-0002-5534-4500, phone +358-50 337 1967

Table S1 Samples and analyses in this study
Day 1 in ”Days from CSTR start” corresponds to the day of the full-scale CSTR reactor start-up

A. Microbial community screening

Process	Process stage	Days from CSTR start	Screening method	Plate counts	Results in	Label in fig or table	Sample description
Etanolix®	hydrolysis	261	amplicon + TRFLP		Fig 1	Hydr start (Day 261)	Etanolix® hydrolysis sample 1 (average operation)
Etanolix®	fermentation	261	amplicon + TRFLP		Fig 1	Ferm end (Day 261)	Etanolix® fermentation sample 1 (average operation)
Etanolix®	raw material	290	amplicon		Fig 1	Raw (Day 290)	Etanolix® raw material sample 1 (poor operation)
Etanolix®	hydrolysis	290	amplicon		Fig 1	Hydr start (Day 290)	Etanolix® hydrolysis sample 2 (poor operation)
Etanolix®	hydrolysis	290	amplicon		Fig 1	Hydr end (Day 290)	Etanolix® hydrolysis sample 3 (poor operation)
Etanolix®	fermentation	290	amplicon		Fig 1	Ferm end (Day 290)	Etanolix® fermentation sample 2 (poor operation)
Etanolix®	raw material	393	amplicon		Fig 1	Raw (Day 393)	Etanolix® raw material sample 2 (good operation)
Etanolix®	hydrolysis	393	amplicon		Fig 1	Hydr end (Day 393)	Etanolix® hydrolysis sample 4 (good operation)
Etanolix®	fermentation	393	amplicon		Fig 1	Ferm start (Day 393)	Etanolix® fermentation sample 4 (good operation)
Etanolix®	fermentation	393	amplicon		Fig 1	Ferm end (Day 393)	Etanolix® fermentation sample 3 (good operation)
Etanolix®	hydrolysis	680	amplicon + TRFLP	TOT, SRB, YEAS, FUNG	Fig 1	Hydr end (Day 680)	Etanolix® hydrolysis sample 5 (average operation)
Etanolix®	fermentation	680	amplicon + TRFLP	TOT, SRB, YEAS, FUNG	Fig 1	Ferm start (Day 680)	Etanolix® fermentation sample 5 (average operation)
Etanolix®	animal feed (side product)	680	amplicon + TRFLP	TOT, SRB, YEAS, FUNG	Fig 1	Animal feed (Day 680)	Etanolix® animal feed sample 2 (average operation)
Bionolix®	fermentation	490	amplicon + TRFLP		Fig 2	Ferm (Day 490)	Bionolix® fermentation sample 1
Bionolix®	hydrolysis	497	amplicon + TRFLP		Fig 2	Hydr (Day 497/499)	Bionolix® hydrolysis sample 1
Bionolix®	fermentation	499	amplicon + TRFLP		Fig 2	Ferm (Day 497/499)	Bionolix® fermentation sample 2
Bionolix®	hydrolysis	504	amplicon + TRFLP		Fig 2	Hydr (Day 504)	Bionolix® hydrolysis sample 2
Bionolix®	fermentation	504	amplicon + TRFLP		Fig 2	Ferm (Day 504)	Bionolix® fermentation sample 3
Bionolix®	hydrolysis	681	amplicon + TRFLP	TOT, SRB, YEAS, FUNG	Fig 2	Hydr (Day 681)	Bionolix® hydrolysis sample 3
Bionolix®	fermentation	681	amplicon + TRFLP	TOT, SRB, YEAS, FUNG	Fig 2	Ferm (Day 681)	Bionolix® fermentation sample 4
Bionolix®	anaerobic reactor effluent	234	amplicon		Fig 3	Bionolix full day 234	Bionolix® anaerobic reactor effluent, day 234
Bionolix®	anaerobic reactor effluent	266	amplicon		Fig 3	Bionolix full day 266	Bionolix® anaerobic reactor effluent, day 266
Bionolix®	anaerobic reactor effluent	338	amplicon		Fig 3	Bionolix full day 338	Bionolix® anaerobic reactor effluent, day 338
Bionolix®	anaerobic reactor effluent	345	amplicon		Fig 3	Bionolix full day 345	Bionolix® anaerobic reactor effluent, day 345

Process	Process stage	Days from CSTR start	Screening method	Plate counts	Results in	Label in fig or table	Sample description
Bionolix®	anaerobic reactor effluent	672	amplicon		Fig 3, Table 3	Bionolix full day 672	Bionolix® anaerobic reactor effluent, day 672
Bionolix®	anaerobic bioreactor lab test	672	amplicon		Fig 3	Bionolix lab day 672	Bionolix® anaerobic bioreactor (10 l) laboratory test sample, testing new process protocol before full-scale implementation
Laboratory	test reactor sludge	616	amplicon		Fig 3	Cellunolix lab start	UASB granular sludge, test reactor 1, beginning of the test (7 liters on IC reactor with simulated Cellunolix® wastewater)
Laboratory	test reactor sludge	674	amplicon		Fig 3	Cellunolix lab not survived	UASB granular sludge, test reactor 3, unsuccessful test where reactor microbes did not survive (7 liters on IC reactor with simulated Cellunolix® wastewater)
Laboratory	test reactor sludge	616	amplicon		Fig 3	Cellunolix lab survived	UASB granular sludge, test reactor 2, successful test (7 liters on IC reactor with simulated Cellunolix® wastewater)
Bionolix®	hydrolysis	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Bionolix® Hydr	Bionolix® hydrolysis sample 4
Bionolix®	anaerobic reactor influent	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Bionolix® Fan	Bionolix® anaerobic reactor influent sample 4
Bionolix®	hydrolysis	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Bionolix® Hydr	Bionolix® hydrolysis sample 5
Bionolix®	fermentation	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Bionolix® Ferm	Bionolix® fermentation sample 5
Bionolix®	anaerobic reactor influent	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Bionolix® Dist	Bionolix® anaerobic reactor influent sample 3
Etanolix®	raw material	1028	TRFLP	LAB	Table 2	Etanolix® 1 Raw	Etanolix® raw material sample 4
Etanolix®	hydrolysis	1028	TRFLP	LAB	Table 2	Etanolix® 1 Hydr start	Etanolix® hydrolysis sample 8
Etanolix®	hydrolysis	1028	TRFLP	LAB	Table 2	Etanolix® 1 Hydr end	Etanolix® hydrolysis sample 9
Etanolix®	fermentation	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Etanolix® 1 Ferm start	Etanolix® fermentation sample 6
Etanolix®	fermentation	1028	TRFLP	LAB	Table 2	Etanolix® 1 Ferm end	Etanolix® fermentation sample 9
Etanolix®	animal feed (side product)	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Etanolix® 1 Animal feed	Etanolix® animal feed sample 3
Etanolix®	raw material	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Etanolix® 2 Raw	Etanolix® raw material sample 3
Etanolix®	hydrolysis	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Etanolix® 2 Hydr start	Etanolix® hydrolysis sample 7
Etanolix®	hydrolysis	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Etanolix® 2 Hydr end	Etanolix® hydrolysis sample 6
Etanolix®	fermentation	1028	TRFLP	LAB	Table 2	Etanolix® 2 Ferm start	Etanolix® fermentation sample 8
Etanolix®	fermentation	881	TRFLP	TOT, LAB, YEAS, FUNG	Table 2	Etanolix® 2 Ferm end	Etanolix® fermentation sample 7
Etanolix®	animal feed (side product)	1028	TRFLP	LAB	Table 2	Etanolix® 2 Animal feed	Etanolix® animal feed sample 4

Plate count abbreviations: TOT = total aerobic bacteria; SRB = sulphate reducing bacteria; LAB = lactic acid bacteria; YEAS = yeasts, FUNG = fungi

B. Small-scale testing of rRNA detection kit

Process	Process stage	Days from CSTR start	HybriScan kits used	Plate counts	Microscopic counts	Results in	Label in fig or table	Sample description
Bionolix®	hydrolysis	1331	TOTAL, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Hydr	Bionolix® hydrolysis sample 6
Bionolix®	fermentation	1331	TOTAL, BEER, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Ferm sample 1	Bionolix® fermentation sample 6
Bionolix®	fermentation	1331	TOTAL, BEER, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Ferm sample 2	Bionolix® fermentation sample 7
Etanolix®	raw material	1331	TOTAL, BEER, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Raw sample 1	Etanolix® raw material sample 5
Etanolix®	raw material	1331	TOTAL, BEER, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Raw sample 2	Etanolix® raw material sample 6
Etanolix®	hydrolysis	1331	TOTAL, BEER, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Hydr start sample 1	Etanolix® hydrolysis sample 10
Etanolix®	hydrolysis	1331	TOTAL, BEER, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Hydr start sample 2	Etanolix® hydrolysis sample 11
Etanolix®	hydrolysis	1331	TOTAL, BEER, YEAST	TOT, LAB, YEAS, FUNG		Fig S1	Hydr end	Etanolix® hydrolysis sample 12
Etanolix®	separation after hydrolysis	1331	TOTAL, BEER	TOT, LAB, YEAS, FUNG		Fig S1	Solid separation sample 1	Etanolix® separation after hydrolysis, liquid fraction
Etanolix®	separation after hydrolysis	1331	TOTAL, BEER	TOT, LAB, YEAS, FUNG		Fig S1	Solid separation sample 2	Etanolix® separation after hydrolysis, solid fraction
Etanolix®	fermentation	1331	TOTAL	TOT, LAB, YEAS, FUNG		Fig S1	Ferm start sampe 1	Etanolix® fermentation sample 10
Etanolix®	fermentation	1331	TOTAL	TOT, LAB, YEAS, FUNG		Fig S1	Ferm start sample 2	Etanolix® fermentation sample 11
Laboratory	fermentation experiment	1459	YEAST	YEAS	yes	Table 4	Separated yeast	Fermentation experiment sample 1 (tests in shake flasks with process yeast)
Laboratory	fermentation experiment	1459	YEAST	YEAS	yes	Table 4	Basic laboratory fermentation	Fermentation experiment sample 2 (tests in shake flasks with process yeast)
Laboratory	fermentation experiment	1459	YEAST	YEAS	yes	Table 4	Late fermentation	Fermentation experiment sample 3 (tests in shake flasks with process yeast)
Laboratory	fermentation experiment	1459	YEAST	YEAS	yes	Table 4	Early fermentation, raw material no 1	Fermentation experiment sample 4 (tests in shake flasks with process yeast)
Laboratory	fermentation experiment	1459	YEAST	YEAS	yes	Table 4	Early fermentation, raw material no 2	Fermentation experiment sample 5 (tests in shake flasks with process yeast)
Laboratory	fermentation experiment	1459	YEAST	YEAS		Table 4	Yeast cream	Fermentation experiment sample 6 (tests in shake flasks with process yeast)

HybriScan kit abbreviations: TOTAL = total bacteria, BEER = lactic acid bacteria, YEAST = spoiling yeasts
Plate count abbreviations: TOT = total aerobic bacteria; LAB = lactic acid bacteria; YEAS = yeasts, FUNG = fungi

C. Full-scale testing of rRNA detection kit

Process	Process stage	Days from CSTR start	HybriScan kits used	Plate counts	Microscopic counts	Results in	Label in fig or table	Sample description
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	fermentor 1 before CIP	Cellunolix® CIP process sample 1
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	fermentor 2 before CIP	Cellunolix® CIP process sample 2
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	fermentor 5 before CIP	Cellunolix® CIP process sample 3
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	hydrolysis tank 1 before CIP	Cellunolix® CIP process sample 4
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	hydrolysis tank 2 before CIP	Cellunolix® CIP process sample 5
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	hydrolysis tank 3 before CIP	Cellunolix® CIP process sample 6
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	hydrolysis tank 4 before CIP	Cellunolix® CIP process sample 7
Cellunolix®	clean-in-place process testing	1849	TOTAL	TOT		Table 5	fermentor 1 after CIP	Cellunolix® CIP process sample 10
Cellunolix®	clean-in-place process testing	1850	TOTAL	TOT		Table 5	fermentor 2 after CIP	Cellunolix® CIP process sample 13
Cellunolix®	clean-in-place process testing	1849	TOTAL	TOT		Table 5	fermentor 3 after CIP	Cellunolix® CIP process sample 11
Cellunolix®	clean-in-place process testing	1848	TOTAL	TOT		Table 5	fermentor 4 after CIP	Cellunolix® CIP process sample 9
Cellunolix®	clean-in-place process testing	1850	TOTAL	TOT		Table 5	fermentor 5 after CIP	Cellunolix® CIP process sample 12
Cellunolix®	clean-in-place process testing	1849	TOTAL	TOT		Table 5	hydrolysis tank 2 after CIP	Cellunolix® CIP process sample 14
Cellunolix®	clean-in-place process testing	1850	TOTAL	TOT		Table 5	hydrolysis tank 3 after CIP	Cellunolix® CIP process sample 15
Cellunolix®	clean-in-place process testing	1850	TOTAL	TOT		Table 5	hydrolysis tank 4 after CIP	Cellunolix® CIP process sample 16
Cellunolix®	yeast separator commissioning	1693	YEAST		yes	Table 6	1st feed to separator	Cellunolix® yeast separator commissioning, sample 1
Cellunolix®	yeast separator commissioning	1693	YEAST		yes	Table 6	1st separated yeast	Cellunolix® yeast separator commissioning, sample 2
Cellunolix®	yeast separator commissioning	1693	YEAST		yes	Table 6	2nd feed to separator	Cellunolix® yeast separator commissioning, sample 3
Cellunolix®	yeast separator commissioning	1693	YEAST		yes	Table 6	2nd separated yeast	Cellunolix® yeast separator commissioning, sample 4
Cellunolix®	yeast separator commissioning	1693	YEAST		estimated based on sample 3	Table 6	3rd feed to separator	Cellunolix® yeast separator commissioning, sample 5
Cellunolix®	yeast separator commissioning	1693	YEAST		yes	Table 6	3rd separated yeast	Cellunolix® yeast separator commissioning, sample 6

HybriScan kit abbreviations: TOTAL = total bacteria, YEAST = spoiling yeasts
Plate count abbreviations: TOT = total aerobic bacteria

Table S2 PCR primers used for the amplicon sequencing approach

	Forward primers	Reverse primers
Archaea	Arch349F GYGCASCAGKCGMGAAW, CGYGCASCAGKCGMGAAW, TRRTGYGCASCAGKCGMGAAW, ACAATTWGYGCASCAGKCGMGAAW (Takai and Horikoshi 2000)	Arch806R GGACTACVSGGGTATCTAAT, TCGGACTACVSGGGTATCTAAT, AACAGGACTACVSGGGTATCTAAT, CTTTCTGTGGACTACVSGGGTATCTAAT (Takai and Horikoshi 2000)
Bacteria	pA AGAGTTTGATCMTGGCTCAG, TAGAGAGTTTGATCMTGGCTCAG, CTCTAGAGTTTGATCMTGGCTCAG (Lane 1991)	pD' GTATTACCGCGGCTGCTG, CGTATTACCGCGGCTGCTG, TAGTATTACCGCGGCTGCTG (Edwards et al. 1989)
Fungi	ITS4 TCCTCCGCTTATTGATATGC, STCCTCCGCTTATTGATATGC, AGTRRTCCTCCGCTTATTGATATGC (White et al. 1990)	fITS7 GTGARTCATCGAATCTTTG, WGTGARTCATCGAATCTTTG, CCAGTGARTCATCGAATCTTTG (Ihrmark et al. 2012)

Table S3 Properties of the full-scale biogas CSTR effluent and collected gas samples. The samples were collected for weekly process monitoring. VFA = volatile fatty acids, DCOD = dissolved chemical oxygen demand, TS = total solids, VS = volatile solids

days	pH	Alkalinity, mg $CaCO_3$/l	VFA, mg/l	VFA / alkalinity	NH_4-N, mgN/g wet wt	DCOD, g/l	TS, %	VS, %	VS / TS	CH_4, %	CO_2, %	O_2, %	H_2S, ppm	NH_3, ppm	CO, ppm	CH_4 / CO_2
1[1)]	8.0	8 713	428	0.05	2.5	2.3	3.9	2.4	0.62							
230	7.7	8 216	566	0.07		3.0				68	32	0.1	121	140	25	2.1
260	7.6	9 411	1 224	0.13		3.8				53	47	0.3	420	298	67	1.1
339	7.3	9 125	7 489	0.82		21.1				59	41	0.0	>500	701	54	1.4
345	6.7	8 893	7 915	0.89	2.4	23.5	5.3	4.2	0.79	58	41	0.3	>500	573	83	1.4
672	7.7	11 400	546	0.05	3.3	2.9	3.8	2.5	0.65	65	34	0.5	38	-	23	1.9

[1)] Day 1 = beginning of the full-scale bioreactor running.

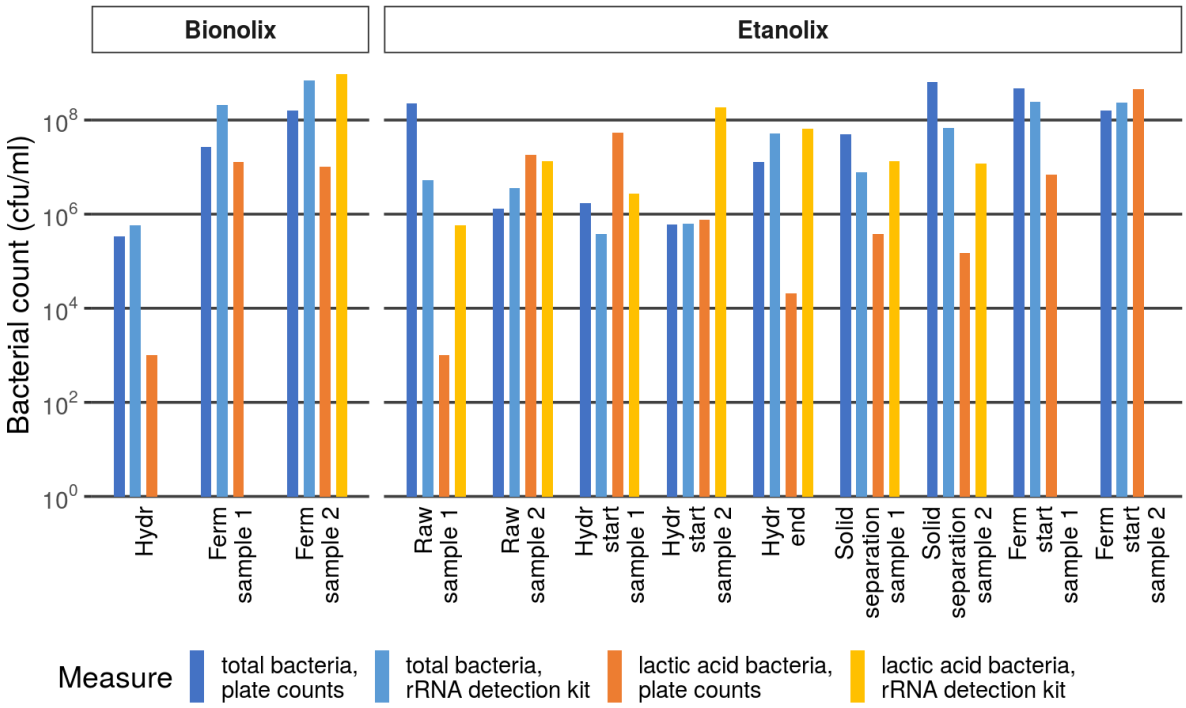

Fig. S1 A comparison of the rRNA detection kit results and the plate counts on PCA (plate count agar) for total bacterial counts and MRS (De Man, Rogosa and Sharpe agar) for lactic acid bacterial counts from the same full-scale bioethanol process samples. Plate counts were done at 30 °C. The rRNA detection kit for lactic acid bacteria was not used for the first two and the last two samples. Sample codes: Raw = feedstock for fermentation, Hydr = hydrolysis, Ferm = fermentation