

Application of Pretreatment, Bioaugmentation and Biostimulation for Fermentative Hydrogen Production from Maize Silage

Vizma Nikolajeva^{1,*}, Miks Neibergs¹, Sintija Valucka¹, Ilze Dimanta¹ and Janis Kleperis²

¹Department of Microbiology and Biotechnology, Faculty of Biology, University of Latvia, Kronvalda blvd. 4, Riga, LV-1586, Latvia

²Laboratory of Hydrogen Energy Materials, Institute of Solid State Physics, University of Latvia, Kengaraga str. 8, Riga, LV-1063, Latvia

Abstract: Bacteria produce hydrogen during anaerobic dark digestion of carbon rich natural resources including renewable cellulosic materials. The purpose of this work was to study the impact of maize silage pretreatment with *Trichoderma* fungi, bioaugmentation with defined bacterial inocula and/ or biostimulation with humic acids and an additional inorganic nitrogen source on the fermentative hydrogen production in laboratory batch assay. Experiments were carried out with and without *Trichoderma asperellum* pretreated silage. The selected bacterial inocula consisted of *Clostridium*, *Enterobacter* and *Tissierella* species, with or without *Bacillus mycoides*. Headspace gas composition, the amount of dry particulate matter, chemical oxygen demand and concentration of volatile fatty acids in liquid were determined. Bacterial communities were studied with fluorescence *in situ* hybridization. The predominant cultivable microbial species were isolated and identified. The study demonstrated a significant increase of hydrogen production from maize silage by indigenous bacteria after pretreatment with *Trichoderma* in comparison with silage untreated with *Trichoderma*. From tested factors, pretreatment, biostimulation with additional nutrients (ammonium nitrate and/ or humic acids) and bioaugmentation with defined bacterial inocula, pretreatment demonstrated significant improvement of hydrogen production from maize silage. Thereby, aerobic treatment with *Trichoderma* could be recommended for the pretreatment of silage for the purpose of fermentative production of hydrogen.

Keywords: Bacteria, bioaugmentation, biostimulation, dark fermentation, hydrogen, maize silage, *Trichoderma*.

INTRODUCTION

Hydrogen is expected to be a widely used energy carrier in the future. Bacteria produce hydrogen using multiple biochemical reactions. Anaerobic digestion is the most studied form of these transformations, when microorganisms use carbon rich natural resources including renewable cellulosic materials. Anaerobic digestion of complex organic matter consists of four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Hydrogen can be produced from acidogenic fermentation and consumed in hydrogenotrophic methanogenesis.

The common biomass categories used in Europe are animal manure and slurry, agricultural residues and by-products, digestible organic wastes from food and agro industries, the organic fraction of municipal waste and catering waste, sewage sludge and dedicated energy crops [1]. Plant materials contain cellulose and lignin. The majority of these macromolecules can be gradually cleaved in aerobic conditions by fungal and bacterial interaction [2].

Before anaerobic digestion, biological pretreatment can be performed by applying either microorganisms or enzymes to the lignocellulose material [3]. The studies using microorganisms comprise mainly those using white- and soft-rot fungi [4] that degrade the lignin fraction as lignin is a barrier to enzymatic hydrolysis of cellulose and also cause unproductive binding of cellulases. The rate of biological treatment is much lower than for most other pretreatment methods and that has for many years been considered to be a factor that prevents industrial use. However, in recent years interest has increased and new studies have been performed involving other types of microorganisms, e.g. brown rot fungi that give a degradation of hemicelluloses and cellulose [5].

Trichoderma species are known as cellulolytic fungi. They contain enzyme complex – endoglucanases, exoglucanases or cellobiohydrolases, and β -glucosidases [2]. However different species, for example *T. reesei*, do not secrete enough β -glucosidase to ensure effective conversion of biomass to low-molecular carbohydrates [6]. Also other plant components such as starch and water-soluble carbohydrates can be used for hydrogen production. For example, feed corn contains 31% starch and 22-25% water-soluble carbohydrates [7] which are used with no difficulty by bacteria in fermentation processes.

*Address correspondence to this author at the Department of Microbiology and Biotechnology, Faculty of Biology, University of Latvia, Kronvalda blvd. 4, Riga, LV-1586, Latvia; Tel/Fax: +371 67034868; E-mail: vizma.nikolajeva@lu.lv

To improve biodegradation and H₂-producing capability, a biostimulation approach has been suggested. Experiments with supplementation of fermentation mixtures with cane molasses [8], waste potato starch and NH₄NO₃ [9] were provided. Studies of nutrient formulations are still necessary for amelioration of hydrogen production.

Galore microbial species, belonging to mesophilic genera *Enterobacter*, *Bacillus*, *Clostridium* [10], *Escherichia*, *Klebsiella*, *Paenibacillus*, *Pantoea* etc. [11] are reported to produce hydrogen through dark fermentation. Enriching H₂-producing pure and mixed microbial cultures from anaerobic substrates, mostly sludge are described in several studies [12-14]. Usually microbial cultures are used for biodegradation experiments with defined and relatively simple model substrates, for example starch [15], xylose [16] or sewage sludge [8]. Nevertheless, microbiologists are still seeking ideal cultures. In our work, bioaugmentation or inoculation of native lignocellulosic substrate with pre-established H₂-producing and H₂-production supporting microorganism association was attempted.

The purpose of this study was to evaluate the influence of maize silage pretreatment with *Trichoderma asperellum* and bioaugmentation with defined bacterial inocula on the hydrogen production in laboratory dark fermentative batch assay. Biostimulation with humic acids and additional inorganic nitrogen source was also analyzed.

MATERIALS AND METHODS

Substrate and Pretreatment of Substrate

Maize silage was obtained from BUFPI Ltd. (Latvia). Pretreatment was carried out with fungus *Trichoderma asperellum* MSCL (Microbial Strain Collection of Latvia) 309. One hundred grams of silage were mixed with 2.5 ml of suspension of *T. asperellum* in a concentration of 10⁹ colony-forming units (cfu) per ml, and solid state fermentation was carried out for three weeks at room temperature.

Batch Experiments

Hydrogen production was studied at 5 g untreated silage (0.76 g dry mass) or 5 g with *Trichoderma asperellum* pretreated silage (1.00 g dry mass) and sterile water or supplements in total volume of 30 ml in 50 ml serum bottles (Supelco Analytical, USA) at 37 °C for 46 days. Bottles were sealed with butyl rubber stoppers (Gotlands Gummifabrik, Sweden) and aluminium crimps (Supelco Analytical, USA), and flushed with argon (AGA Ltd., Latvia).

The following 11 variants were created:

- C – untreated silage;
- H – untreated silage (C) supplemented with 0.3 ml of humic acid preparation (kindly provided by Faculty of Geography and Earth Sciences, University of Latvia);
- B – untreated silage (C) supplemented with 0.6 ml of selected bacterial inoculum consisting of 5.6 log cfu of *Clostridium butyricum* MSCL 1019, 5.6 log cfu of *Clostridium paraputrificum* MSCL 1171, 6.3 log cfu of

Enterobacter asburiae MSCL 899, 3.8 log cfu of *E. cloacae* MSCL 1166, 5.8 log cfu of *Tissierella praeacuta* MSCL 1160 and 7.6 log cfu of *Bacillus mycoides* MSCL 1010;

HB – variant H supplemented with bacterial inoculum as in variant B;

T – pretreated silage;

TH – pretreated silage (T) supplemented with 0.3 ml of humic acid preparation;

TN – pretreated silage (T) supplemented with a solution of NH₄NO₃ to a final concentration of 5 g/l;

TB – pretreated silage (T) supplemented with selected bacterial inoculum as in variant B;

TB-B – pretreated silage (T) supplemented with bacterial inoculum as in variant B but without *Bacillus mycoides* MSCL 1010;

TNB – variant TN supplemented with bacterial inoculum as in variant B;

THB – variant TH supplemented with bacterial inoculum as in variant B.

The presented value in this study was the average value of the duplication.

Methods of Chemical Analyses

Evolved gases from the headspace of the bottles in the amount of 10 cm³ were collected with a graduated, gas-tight syringe from the test system and tested in the mass-spectrometer RGAPro-100 (Setaram Instrumentation, France). The RGAPro is a residual gas analyzer designed to work with the PCTPro-2000 or any other gas process for real-time compositional analysis of gas sorption and reaction processes. The system uses a 100 atomic mass units (amu) quadrupole mass spectrometer with better than 1 amu resolution. The unit for mass spectra graphic on x-axis is *m/z*, where *m* is amu of measured molecule and *z* is the charge. The data from mass spectrometer were analyzed by RGA 3.0 Software for SR Residual Gas Analysers program [17].

The liquid samples taken from the bottles were centrifuged at 5000 rpm for 10 min, and then the supernatants were filtered through 0.45 μm cellulose acetate membranes and mixed with acetonitril (SAFC, USA) in ratio 1:1. The concentration of the volatile fatty acids (VFAs) was determined using modular UPLC system Agilent 1290 *Infinity* series (Agilent Technologies, Germany). HPLC column with a dimension of 4.6 x 150 mm (Atlantis T3, 3 μm, Waters, Ireland) was placed in line from the LC system and attached directly to the 1290 *Infinity* DAD detector (Agilent Technologies, Germany). All samples were scanned over an UV range of 200-400 nm. A flow rate of 0.4 ml/min was maintained for all sample analyses. Elution solvents consist of 1 mM sulfuric acid and acetonitril in 12 minutes gradient mode. Acetonitril (HPLC grade) was supplied by LiChrosolv (Merck KGaA, Germany) and sulphuric acid (99.999%) was supplied by Sigma-Aldrich (St. Louis, USA). The water used was purified with a Milli-Q water purification system from Millipore (Bedford, USA). Data

were obtained and processed with MassHunter system (Agilent Technologies, USA).

Chemical Oxygen Demand (COD) was estimated in supernatants after centrifugation of liquids at 10000 rpm for 5 min. using RD 125 Thermoreactor (Lovibond, Germany) and MD200 Photometer (Lovibond, Germany).

The pH values were measured using a pH meter AD 1405 (Adrona, Latvia).

Isolation and Identification of Culturable Microorganisms

Bacteria were isolated in agar plates with R2A medium (SIFIN, Germany) at a temperature of 37 °C. Serial decimal dilutions of samples were incubated aerobically or anaerobically (GasPak Anaerobe Pouch, Becton & Dickinson, USA). The cultivation was repeated until a single morphology was detected by microscopic observation. Isolated pure cultures were identified biochemically with BBL Crystal ID kits for Gram-Positive bacteria, Enteric/ Nonfermenter bacteria and Anaerobe (Becton & Dickinson, USA).

Fluorescence in situ Hybridization (FISH)

The basic method described by Pernthaler *et al.* [18] and Fuchs *et al.* [19] was used. The samples were air-dried, fixed in 4% para-formaldehyde for 1.5-2 hours. After drying, the samples were dehydrated in ethanol (50, 80, and 96%). The following 16S rRNA-targeted oligonucleotide probes were used: LGC353B, GAM42a and Chis150 (Table 1). The formamide concentration for the hybridization (2 h, 46 °C) and NaCl concentration for the washing (20 min, 48 °C) are listed in Table 1. The probes, marked at their 5'-end with Cy3, were purchased from Eurofins MWG Operon (Germany). Epifluorescent microscope DM 2000 (Leica Microsystems, Germany) was used for examination of hybridization results.

Table 1. Oligonucleotide probes used in FISH analyses.

Probe	Specificity	Sequence (5' – 3')	Formamide ^a (%) / NaCl ^b (mM)	Reference
LGC353B	<i>Bacillus</i> spp.	GCG GAA GAT TCC CTA CTG C	35 / 215	[20]
GAM42a	Gamma-proteobacteria	GCC TTC CCA CAT CGT TT	0 / 70	[21]
Chis150	<i>Clostridium</i> clusters	TTA TGC GGT ATT AAT CTY CCT TT	20 / 900	[22]

^a – Formamide concentration in the hybridization buffer.

^b – NaCl concentration in the washing buffer.

Table 2. Change of pH values during fermentation. Untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae*, *Tissierella praeacuta* and *Bacillus mycoides*, or five bacteria (B-B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae* and *Tissierella praeacuta*. Standard deviation did not exceed 0.01.

Day	Variants										
	C	H	B	HB	T	TH	TN	TB	TB-B	TNB	THB
0	6.55				6.44						
46	4.21	4.40	4.36	4.32	4.98	4.74	5.11	4.80	4.97	5.45	5.00

SEM

Air-dried samples were treated with solution of 96% ethanol and diethyl ether in the ratio 1:1 and analysed using scanning electron microscope Mira/LMU (Tescan, Czech Republic).

RESULTS

Production of Gases

In fermentation variants with untreated silage 1-2% of hydrogen gas was produced but release of hydrogen in the amount of 15-21% from headspace gases was established in all variants with silage pretreated with fungus *Trichoderma asperellum* (Fig. 1). Low H₂ production (≤ 0.3%) was observed in bioaugmentation variants with six bacteria (TB) but the variant with five bacteria (TB-B) showed ten times higher production after 7 and 28 days of incubation. Maximum hydrogen production was observed after 28 days of incubation in all of the variants with the exception of TB. Delayed and prolonged release of hydrogen was detected in TN and TNB, where pretreated silage was supplemented with inorganic nitrogen.

The proportion of carbon dioxide reached 40-43% from the volume of headspace gases and the ratio of hydrogen and carbon dioxide reached 1:2. Dynamic of CO₂ production (Fig. 2) resembled dynamic of H₂ production. Pearson's correlation coefficients r=0.9509 (p <0.00001) and r=0.8931 (p <0.00021), on the 7th and 28th day respectively, indicated a strong positive relationship between the values of CO₂ and H₂.

Concentration of methane was below 0.0% from gases in all cases.

Biodegradation of Silage

SEM analyses demonstrated the effect of fungal pretreatment on the structure of maize silage. Fig. (3A)

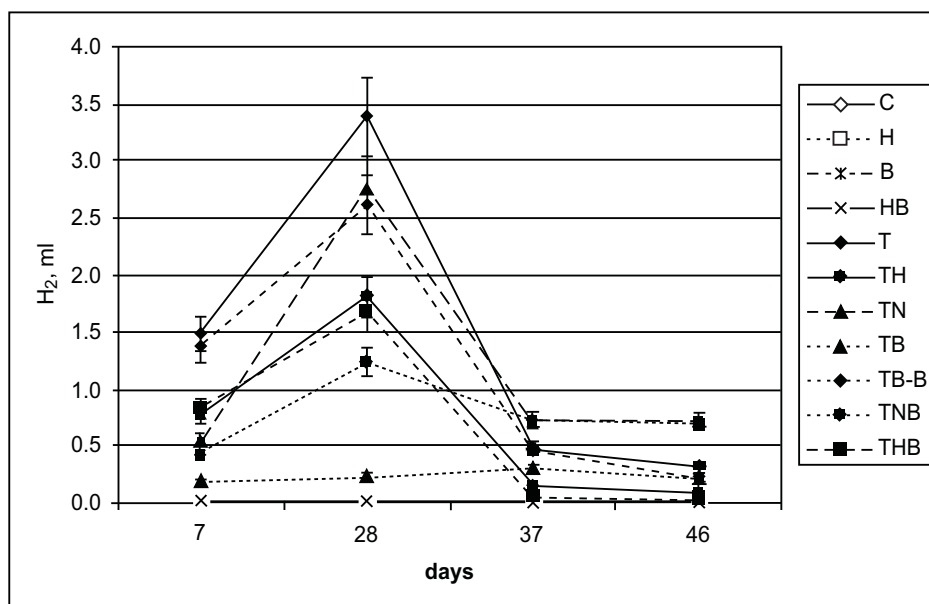


Fig. (1). Biohydrogen production during fermentation of untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae*, *Tissierella praeacuta* and *Bacillus mycooides*, or five bacteria (B-B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae* and *Tissierella praeacuta*.

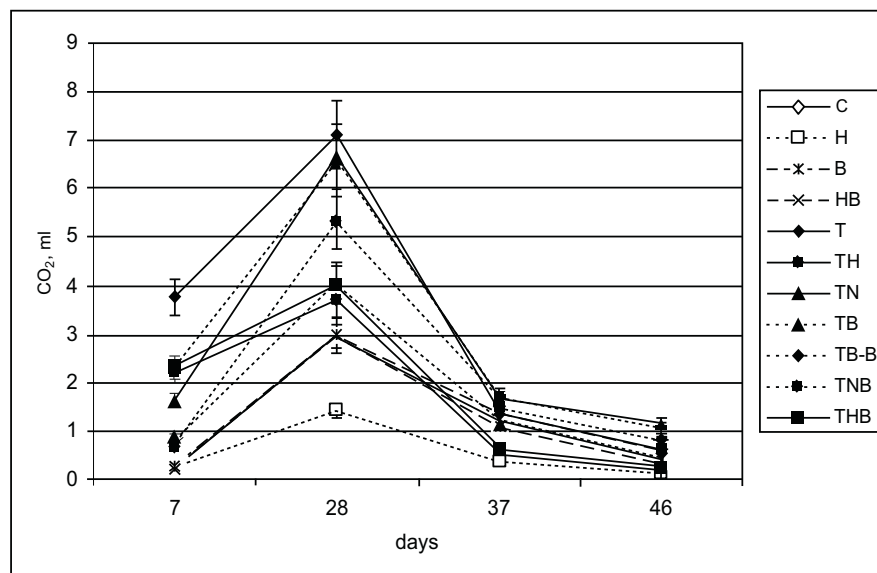


Fig. (2). Carbon dioxide production during fermentation of untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae*, *Tissierella praeacuta* and *Bacillus mycooides*, or five bacteria (B-B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae* and *Tissierella praeacuta*.

shows silage without pretreatment and there we can see relatively little affected plant surface whereas Fig. (3B) shows pretreated silage with partially degraded cell walls.

Loss of dry mass during fermentation was established. Fig. (4) shows the proportion of consumed amount of dry mass of silage in comparison with initial input in all variants. Fungal pretreatment increased the loss of dry mass during fermentation for an average 13%. Moreover, the loss during fungal pretreatment was 24% and this parameter was not taken into account in the following calculations. Humic acid

supplement significantly ($p < 0.05$) increased the consumption of silage in untreated variant H but bacterial inoculums decreased the consumption in variants B and TNB in comparison with variants C and T ($p < 0.05$).

Chemical Changes During Fermentation

To find out the amount of remaining dissolved organic matter at the end of the experiment, COD was estimated. Obtained results (Fig. 5) show that variants without silage pretreatment contained on average 30% higher concentration

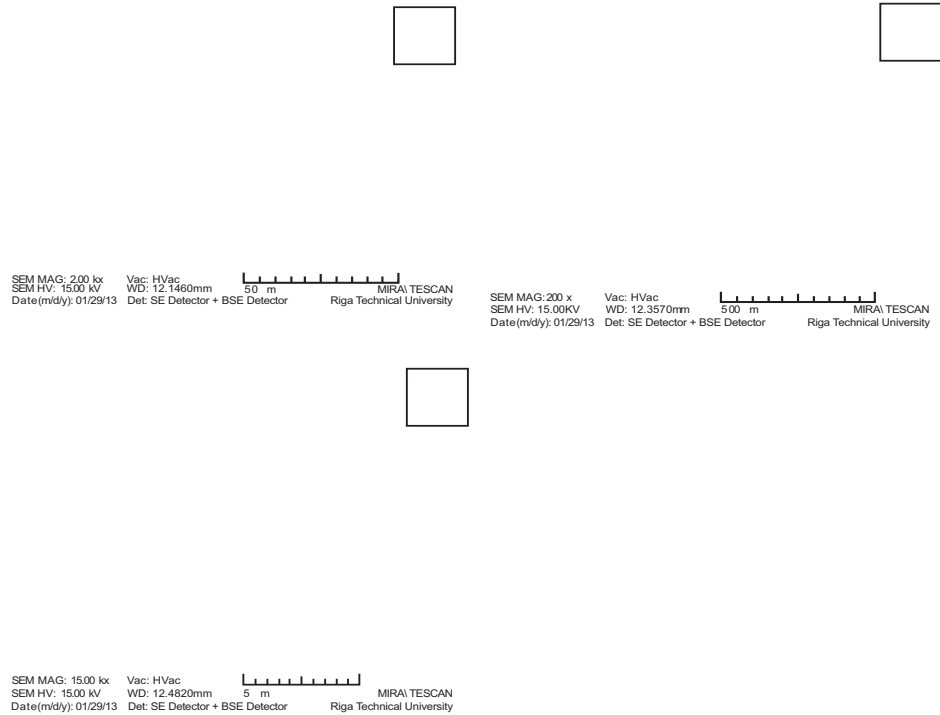


Fig. (3). SEM image of maize silage without pretreatment (A), pretreated with *Trichoderma asperellum* during three weeks (B), and pretreated silage after fermentation, with visible warty conidia of *T. asperellum* (C).

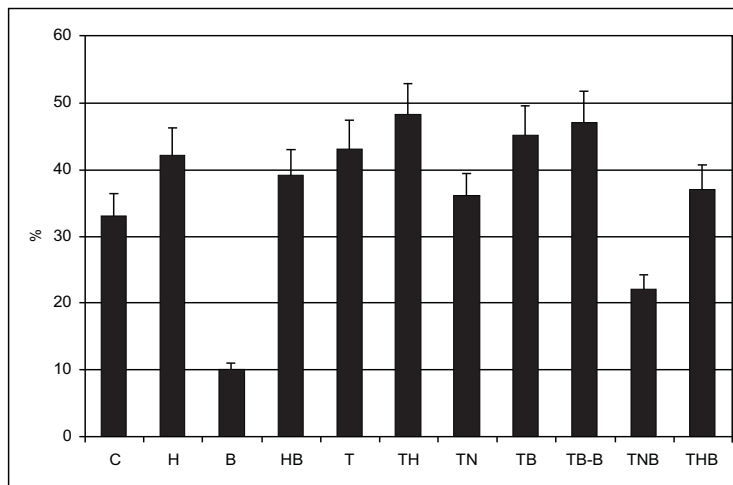


Fig. (4). Proportion of consumed amount of dry mass of silage during fermentation in comparison with initial input. Untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae*, *Tissierella praeacuta* and *Bacillus mycoides*, or five bacteria (B-B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae* and *Tissierella praeacuta*.

of COD than variants with pretreated silage. Weak negative correlation $r = -0.4691$ ($p = 0.1456$) was found between COD (Fig. 5) and the proportion of consumed dry mass (Fig. 4).

Initial pH in the fermentation medium was 0.11 lower after fungal pretreatment than without pretreatment. During anaerobic fermentation, pH values decreased in all variants (Table 2) especially in untreated silage where pH reached 4.21 in variant C. The pH values ranged from 5.45 in TNB to 4.74 in TH in the pretreated silage.

HPLC analyses provided information on the concentration of important organic acids – formic acid, acetic acid, lactic acid, propionic acid and butyric acid – in liquid at the end of fermentation. Data summarized in Table 3 show that pretreatment of silage resulted in decrease of the total concentration of organic acids for 10.5-36.9% in comparison with pretreated silage in all comparable variants. The proportion of formic acid varied from 3.0% in B to 5.9% in TB-B. Concentration of acetic acid was low and anywhere exceeded 0.2%. Proportion of lactic acid and propionic acid

Table 4. Bacteria determined by FISH and predominant bacteria isolated from fermentation liquids and identified biochemically with BBL Crystal ID kits. Untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae*, *Tissierella praeacuta* and *Bacillus mycoides*, or five bacteria (B-B): *Clostridium butyricum*, *C. paraputrificum*, *Enterobacter asburiae*, *E. cloacae* and *Tissierella praeacuta*. + - detected by FISH; - undetected by FISH; LGC353B – probe for *Bacillus* spp.; GAM42a – probe for Gamma-proteobacteria; Chis150 – probe for *Clostridium* clusters.

Variant	BBL Crystal ID results			FISH results with probe		
	Species	Biotype validity	Confidence	LGC353B	GAM42a	Chis150
C	<i>Bacillus brevis</i>	121	0.9999	+	-	-
	<i>Bacillus licheniformis</i>	136	0.9986			
	<i>Lactobacillus johnsonii</i>	1092957	0.9700			
H	<i>Propionibacterium avidum</i>	1000000	0.9333	-	-	-
B	<i>Propionibacterium propionicum</i>	548859	0.9608	-	-	-
HB	<i>Propionibacterium avidum</i>	1000000	0.9333	-	-	-
	<i>Lactobacillus johnsonii</i>	1092957	0.9700			
	<i>Propionibacterium propionicum</i>	548859	0.9608			
T	<i>Bacillus megaterium</i>	71205	0.9995	+	-	+
	<i>Propionibacterium avidum</i>	1000000	0.9333			
	<i>Clostridium beijerinckii</i>	4183	0.9989			
TH	<i>Propionibacterium avidum</i>	1000000	0.9333	-	-	+
	<i>Clostridium beijerinckii</i>	4183	0.9989			
TN	<i>Rothia mucilaginosa</i>	166	0.9999	-	-	+
	<i>Clostridium beijerinckii</i>	4183	0.9989			
TB	<i>Bacillus brevis</i>	5	0.9999	+	-	-
	<i>Bacillus mycoides</i>	413	0.9995			
	<i>Propionibacterium propionicum</i>	548859	0.9608			
TB-B	<i>Propionibacterium avidum</i>	1000000	0.9333	+	-	+
	<i>Bacillus brevis</i>	5	0.9999			
	<i>Clostridium beijerinckii</i>	4183	0.9989			
	<i>Lactobacillus johnsonii</i>	1092957	0.9700			
TNB	<i>Leifsonia aquatica</i>	166	0.9999	+	-	+
	<i>Bacillus brevis</i>	121	0.9999			
	<i>Clostridium beijerinckii</i>	4183	0.9989			
THB	<i>Propionibacterium propionicum</i>	548859	0.9608	-	-	+
	<i>Clostridium beijerinckii</i>	4183	0.9989			
	<i>Bacillus licheniformis</i>	136	0.9986			

Detected Microorganisms

FISH analyses of fermented liquids were provided with three oligonucleotide probes and indicated the presence of *Clostridium* clusters I and II including *C. beijerinckii* in all pretreated variants without TB, presence and dominance of

Bacillus spp. in five variants (C, T, TB, TB-B and TNB), and absence of Gamma-proteobacteria (Table 4).

From one to four predominant species of bacteria from every variant were isolated and identified (Table 4). From six bioaugmentation variants, *Bacillus mycoides*, one of the inoculated bacteria, was isolated in one case, from TB. Other

inoculated bacterial species failed to become predominant. Three indigenous *Bacillus* species, i.e., *B. brevis* (synonym *Brevibacillus brevis*), *B. licheniformis* and *B. megaterium*, were isolated from variants C, T, THB, TB and TB-B. *Lactobacillus johnsonii* was a sole isolated lactic acid bacterium and it was found in three variants (C, HB and TB-B). Eight variants contained *Propionibacterium* spp. and six variants, all belonging to pretreated silage, contained *Clostridium beijerinckii*. We failed to isolate any fungus from fermented silage despite *Trichoderma asperellum* conidia in SEM (Fig. 3C) in pretreated silage.

DISCUSSION

Renewable cellulosic materials are known as a sustainable source for biofuel production. This study demonstrated the feasibility of biohydrogen production by dark fermentation of maize silage at temperature of 37 °C. The provided 46 day long experiments showed that indigenous bacteria induce loss of dry mass from 10% to 42% (Fig. 4) and produce a little amount of carbon dioxide (Fig. 2) and almost no hydrogen (Fig. 1) from native, untreated silage. At the same time, the decrease of pH from 6.55 to 4.40-4.21 and the accumulation of fatty acids, in particular propionic acid and butyric acid, was observed (Table 3). Predominant *Propionibacterium* species producing propionic acid were isolated from variants H, B and HB (Table 4). Khanal et al. [23] found that the specific hydrogen production rate is the highest for the pH range of 5.5-5.7. When pH drops to 4.5 or below, the clostridial populations reach the stationary growth phase and the reactions shift from a hydrogen/ acid production phase to a solvent production phase [24]. In addition, the optimum pH for methanogenesis is between pH 6.5 and pH 8.0 [25] therefore we suppose that methanogenesis was inhibited in our experiments due to low pH values. Obtained data allow assume that microbial activities became exhausted on day 37. The remaining COD indicated on the unutilized soluble compounds including volatile fatty acids, mainly butyric acid and propionic acid, which were not be able to serve for substrates in particular conditions.

Silage pretreatment with fungus *Trichoderma asperellum* for three weeks at room temperature strengthened fermentation and releasing of hydrogen and carbon dioxide what indicates fermentative activities [26]. The amount of consumed dry mass exceeded 45% in four pretreated variants THB, T, TB and TB-B. However, the difference of hydrogen production was high among these variants. No variant of bioaugmentation (TB, TB-B) or bioaugmentation plus biostimulation (THB) exceeded control variant T in relation to hydrogen production as well carbon dioxide production. In the case of active fermentation (TB-B, TN, THB, T, and TH), the volume of hydrogen reached 29-33% of the total gas volume. Variant T also demonstrated the least remaining COD in the solution after fermentation (Fig. 5). One of the predominant bacteria was *Clostridium beijerinckii* in pretreated variants with the exception of TB which demonstrated a low level of hydrogen production. It is known that *C. beijerinckii* belongs to hydrogen-producing organisms [26-28]. Fungi *Trichoderma asperellum* inoculated in silage at the beginning of aerobic pretreatment

procedure had lost their viability after 46 days of anaerobic fermentation.

To enhance hydrogen gas production, Kim et al. [29] reported the addition of nitrate (KNO_3 1 g/l) for methanogenesis inhibition. Our efforts to promote hydrogen production with an additional nitrogen source, ammonium nitrate, did not lead to the expected results. Either nitrogen was in sufficient quantities in the silage, or there was too much concentration of additional nitrate, 5 g/l. For comparison, Wang and Jin [9] have described process optimization of biohydrogen production from molasses by using 1.2 g/l NH_4NO_3 . However, we observed metabolic changes in variants with additional nitrogen, i.e., decrease of the proportion of lactic acid to zero, decrease of propionic acid to 2.8-8.0%, as well as an increase of formic acid to 5.8-6.7% and in particular, an increase of butyric acid to 85-91%. An excess nitrogen has been found to promote the production of higher organic acids and ethanol, resulting in lowering hydrogen production efficiency [30].

The addition of humic acids in silage reduced the release of hydrogen in all the studied variants and decreased ($p < 0.05$) the proportion of the consumed amount of dry mass in variants without pretreatment (Fig. 4). It is known that diversity of bacteria can utilize humic substances as electron donors for anaerobic respiration [31], compete with methanogenesis [32] and suppress hydrogenotrophic methanogenesis [33]. As confirmed in our study, an abundance of humic acids adversely affects the production of hydrogen.

Some aerobic *Bacillus* species and facultative anaerobic *Enterobacteriaceae* are known as hydrogen producers and anaerobic environment creators [34]. Chang et al. [35] suggested contribution of *Bacillus* and *Clostridium* co-culture for development of industrialized bio-fuels and biohydrogen producing systems from biomass. Our attempt to make an effective bacterial community for hydrogen production from maize silage did not achieve the expected aim. Bioaugmentation of silage with defined bacterial inocula consisting of anaerobic (*Clostridium butyricum*, *C. paraputrificum*, *Tissierella praeacuta*) and facultative anaerobic bacteria (*Enterobacter asburiae*, *E. cloacae*) with or without aerobic *Bacillus mycoides* did not increase hydrogen (Fig. 1) and CO_2 production (Fig. 2) and amount of consumed dry mass (Fig. 4). Although inoculated bacteria failed to become predominant, with the exception of *Bacillus mycoides* in TB, their effect was unfavorable for fermentation processes. In particular, adverse effects were shown by *B. mycoides*. If we compare concentration of organic acids at the end of fermentation, variant TB showed greater amount of total fatty acids than TB-B. Exactly, concentration of lactic acid and propionic acid was increased in the presence of *B. mycoides*. To our best knowledge, there are no investigations about anaerobic metabolism of this species but it is known that its close relatives can grow anaerobically by respiration with nitrate as a terminal electron acceptor and produce lactate, acetate and 2,3-butanediol as the major anaerobic fermentation products [36]. Screening of 203 *Bacillus* natural isolates provided by Beric et al. [37] demonstrated that 127 strains exhibit antimicrobial activity. Accordingly, special attention should

be paid to the selection of appropriate *Bacillus* species in future experiments.

Acetate is the most important intermediate in anaerobic environments but acetate constituted only 0.0-0.2% of total volatile fatty acids at the end of fermentation (Table 3). We assume that acetate has been utilized. The ability to degrade acetate is widely spread among methanogenic, sulfate-reducing and nitrate-reducing microorganisms [38]. Methane was not found in our analyses therefore we suppose that our fermentation liquids contained active acetate-utilizing and nitrate-reducing bacteria. Elefsiniotis *et al.* [39] investigated the ability of naturally-produced volatile fatty acids act as a carbon source for the removal of nitrate and found that the denitrifier population had a preference for acetic acid, and butyric acid and propionic acid were consumed only after acetate concentrations began to decline. According to investigation of S. F. Magram [40], formic acid is not a good carbon source for denitrification.

CONCLUSION

The present study was an attempt to improve the biohydrogen production using various nutrient and seed formulation methods on silage. From tested factors, pretreatment, biostimulation with additional nutrients (ammonium nitrate and/ or humic acids) and bioaugmentation with defined bacterial inocula, pretreatment demonstrated significant improvement of hydrogen production from maize silage. Thereby, aerobic pretreatment with *Trichoderma asperellum* MSCL 309 and possibly other species and strains of fungi from genus *Trichoderma* could be recommended for the pretreatment of silage for the purpose of fermentative production of hydrogen.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to Ph.D. Ilva Nakurte for help with performing liquid chromatography analyses. This work was supported by the National Research Programme of Latvia LATENERGI project "Research of hydrogen extraction, storage and energy production methods and elaboration of prototypes" and Latvian Council of Science project No. 666/2014 "Synthesis and studies on controlled porosity composite thin layers and systems for energy storage and conversion applications".

REFERENCES

- [1] Al Seadi T, Rutz D, Prassl H, *et al.* Eds. Biogas handbook. Esbjerg: University of Southern Denmark 2008; pp. 125.
- [2] Yang B, Dai Z, Ding SY, Wuman CE. Enzymatic hydrolysis of cellulosic biomass. *Biofuels* 2011; 2: 421-50.
- [3] Lee J. Biological conversion of lignocellulosic biomass to ethanol. *J Biotechnol* 1997; 56: 1-24.
- [4] Belkacemi K, Turtotte G, de Halleux D, Savoie P. Ethanol production from AFEX-treated forages and agricultural residues. *Appl Biochem Biotechnol* 1998; 70-72: 441-62.
- [5] Ray MJ, Leak DJ, Spanu PD, Murphy RJ. Brown rot fungal early stage decay mechanism as a biological pretreatment for softwood

- biomass in biofuel production. *Biomass Bioenergy* 2010; 34: 1257-62.
- [6] Berlin A, Maximenko V, Gilkes N, Saddler J. Optimization of enzyme complexes for lignocellulose hydrolysis. *Biotechnol Bioeng* 2007; 97: 287-96.
- [7] Martinez-Perez N, Cherrymann S, Premier G, *et al.* The potential for hydrogen enriched biogas production from crops: scenarios in the UK. *Biomass Bioenergy* 2007; 31: 94-105.
- [8] Kotay SM, Das D. Novel dark fermentation involving bioaugmentation with constructed bacterial consortium for enhanced biohydrogen production from pretreated sewage sludge. *Int J Hydrogen Energy* 2009; 34: 7489-96.
- [9] Wang X, Jin B. Process optimization of biological hydrogen production from molasses by a newly isolated *Clostridium butyricum* W5. *J Biosci Bioeng* 2009; 107: 138-44.
- [10] Nandi R, Sengupta S. Microbial production of hydrogen: an overview. *Crit Rev Microbiol* 1998; 24: 61-84.
- [11] Kanso S, Dasri K, Tingthong S, Watanapokasin RY. Diversity of cultivable hydrogen-producing bacteria isolated from agricultural soils, waste water sludge and cow dung. *Int J Hydrogen Energy* 2011; 36: 8735-42.
- [12] Chen WM, Tseng ZJ, Lee KS, Chang JS. Fermentative hydrogen production with *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge. *Int J Hydrogen Energy* 2005; 30: 1063-70.
- [13] Kotay SM, Das D. Microbial hydrogen production with *Bacillus coagulans* IIT-BT S1 isolated from anaerobic sewage sludge. *Bioresour Technol* 2007; 98: 1183-90.
- [14] Yang M, Yu HQ, Wang G. Evaluation of three methods for enriching H₂-producing cultures from anaerobic sludge. *Enzyme Microb Technol* 2007; 40: 947-53.
- [15] Lee KS, Hsu YF, Lo YC, Lin PJ, Lin CY, Chang JS. Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *Int J Hydrogen Energy* 2008; 33: 1565-72.
- [16] Wu SY, Lin CY, Lin PJ, *et al.* Dark fermentative hydrogen production from xylose in different bioreactors using sewage sludge microflora. *Energy Fuels* 2008; 22: 113-9.
- [17] RGAPro for the PCTPro-2000 sorption gas analyzer. Developed by Hy-Energy Scientific, available from SETARAM Instrumentation (Caluire, France). Accessed August 08, 2014, at: <http://www.setaram.com/RGAPro.htm>.
- [18] Pernthaler J, Glöckner FO, Schönhuber W, Amann R. Fluorescence *in situ* hybridization with rRNA-targeted oligonucleotide probes. *Method Microbiol* 2001; 30: 207-27.
- [19] Fuchs BM, Pernthaler J, Amann R. In: Reddy CA, Beveridge TJ, Breznak JA, Marzluf G, Schmidt TM, Snyder LR, Eds. *Single Cell Identification by Fluorescence in Situ Hybridization. Methods for general and molecular microbiology*, 3rd ed., Washington: ASM Press, 2007; 886-96.
- [20] Felske A, Akkermans ADL, De Vos WM. *In situ* detection of an uncultured predominant *Bacillus* in Dutch grassland soils. *Appl Environ Microbiol* 1998; 64: 4588-90.
- [21] Manz W, Amann R, Ludwig W, Wagner M, Schleifer KH. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Syst Appl Microbiol* 1992; 15: 593-600.
- [22] Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, Welling GW. Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 1998; 64: 3336-45.
- [23] Khanal SK, Chen WH, Sung S. Biological hydrogen production: effects of pH and intermediate products. *Int J Hydrogen Energy* 2004; 29: 1123-31.
- [24] Byung HK, Zeikus JG. Importance of hydrogen metabolism in regulation of solventogenesis by *Clostridium acetobutylicum* continuous culture system of hydrogen-producing anaerobic bacteria. *Proceedings 8th International Conference on Anaerobic Digestion* 1985; vol. 2: pp. 383-90.
- [25] Mohan SV, Mohanakrishna G, Sarma PN. Integration of acidogenic and methanogenic processes for simultaneous production of biohydrogen and methane from wastewater treatment. *Int J Hydrogen Energy* 2008; 33: 2156-66.
- [26] Barlaz MA, Schaefer DM, Ham RK. Bacterial population development and chemical characteristics of refuse decomposition

- in a simulated sanitary landfill. *Appl Environ Microbiol* 1989; 55: 55-65.
- [27] Lin PY, Whang LM, Wu YR, *et al.* Biological hydrogen production of the genus *Clostridium*: metabolic study and mathematical model simulation. *Int J Hydrogen Energy* 2007; 32: 1728-35.
- [28] Skonieczny MT, Yargeau V. Biohydrogen production from wastewater by *Clostridium beijerinckii*: effect of pH and substrate concentration. *Int J Hydrogen Energy* 2009; 34: 3288-94.
- [29] Kim JO, Kim YH, Yeom SH, Song BK, Kim IH. Enhancing continuous hydrogen gas production by the addition of nitrate into an anaerobic reactor. *Process Biochem* 2006; 41: 1208-12.
- [30] Sreethawong T, Chatsiriwatana S, Rangsunvigit P, Chavadej S. Hydrogen production from cassava wastewater using an anaerobic sequencing batch reactor: Effects of operational parameters, COD:N ratio, and organic acid composition. *Int J Hydrogen Energy* 2010; 35: 4092-102.
- [31] Coates JD, Cole KA, Chakraborty R, O'Connor SM, Achenbach LA. Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. *Appl Environ Microbiol* 2002; 68: 2445-52.
- [32] Cervantes FJ, Van der Velde S, Lettinga G, Field JA. Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiol Ecol* 2000; 34: 161-71.
- [33] Klupfel L, Piepenrock A, Kappler A, Sander M. Humic substances as fully regenerable electron acceptors in recurrently anoxic environments. *Nat Geosci* 2014; 7: 195-200.
- [34] Chang JJ, Chen WE, Shih SY, *et al.* Molecular detection of the clostridia in an anaerobic biohydrogen fermentation system by hydrogenase mRNA-targeted reverse transcription-PCR. *Appl Microbiol Biotechnol* 2006; 70: 598-604.
- [35] Chang JJ, Chou CH, Ho CY, Chen WE, Lay JJ, Huang CC. Syntrophic co-culture of aerobic *Bacillus* and anaerobic *Clostridium* for bio-fuels and bio-hydrogen production. *Int J Hydrogen Energy* 2008; 33: 5137-46.
- [36] Ramos HC, Hoffmann T, Marino M, *et al.* Fermentative metabolism of *Bacillus subtilis*: physiology and regulation of gene expression. *J Bacteriol* 2000; 182: 3072-80.
- [37] Beric T, Kojic M, Stankovic S, *et al.* Antimicrobial activity of *Bacillus* sp. natural isolates and their potential use in the biocontrol of phytopathogenic bacteria. *Food Technol Biotech* 2012; 50: 25-31.
- [38] Scholten JCM, Stams AJM. Isolation and characterization of acetate-utilizing anaerobes from a freshwater sediment. *Microb Ecol* 2000; 40: 292-9.
- [39] Elefsiniotis P, Wareham DG, Smith MO. Use of volatile fatty acids from an acid phase digester for denitrification. *J Biotechnol* 2004; 114: 289-97.
- [40] Magram SF. Drinking water denitrification in a packed bed anoxic reactor: effect of carbon source and reactor depth. *J Appl Sci* 2010; 10: 558-63.

Received: July 22, 2014

Revised: November 06, 2014

Accepted: November 10, 2014

© Nikolajeva *et al.*; Licensee Bentham Open.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.