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

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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* hibridization. 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.

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To improve biodegradation and H_2-producing capability, a biostimulation approach has been suggested. Experiments with supplementation of fermentation mixtures with cane molasses [8], waste potato starch and NH_4NO_3 [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 H_2-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 H_2-producing and H_2-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 10^9 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 NH_4NO_3 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 cm^3 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-ass 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.

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 H_2 production ($\leq$ 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 CO_2 production (Fig. **2**) resembled dynamic of H_2 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 CO_2 and H_2.

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**)

Table 1. Oligonucleotide probes used in FISH analyses.

Probe	Specificity	Sequence (5' – 3')	Formamide[a] (%) / NaCl[b] (mM)	Reference
LGC353B	*Bacillus* spp.	GCG GAA GAT TCC CTA CTG C	35 / 215	[20]
GAM42a	Gamma-proteobacteria	GCC TTC CCA CAT CGT TT	0 / 70	[21]
Chis150	*Clostridium* clasters	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

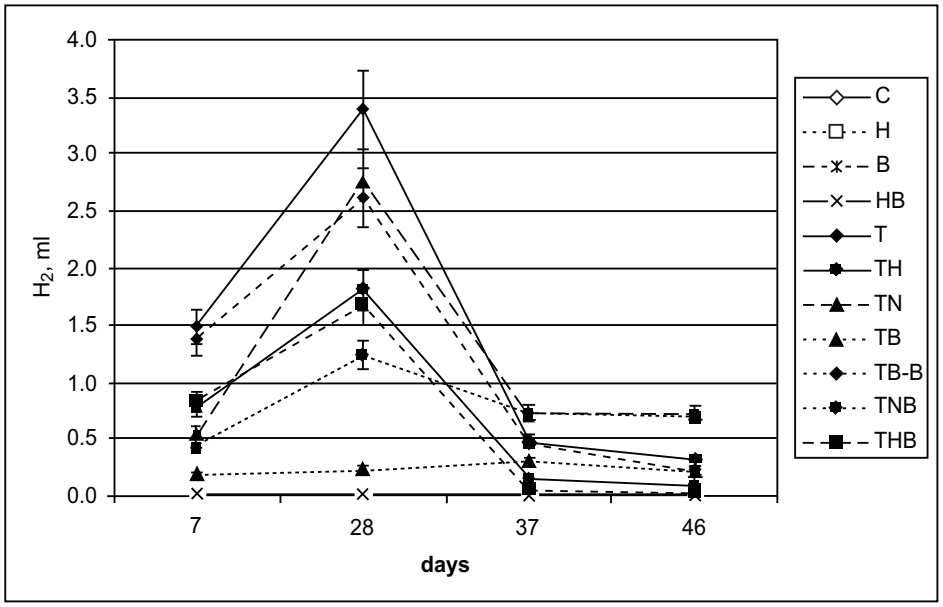

Fig. (1). Biohydrogen production during fermentation of untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum, C. paraputrificum, Enterobacter asburiae, E. cloacae, Tissierella praeacuta* and *Bacillus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

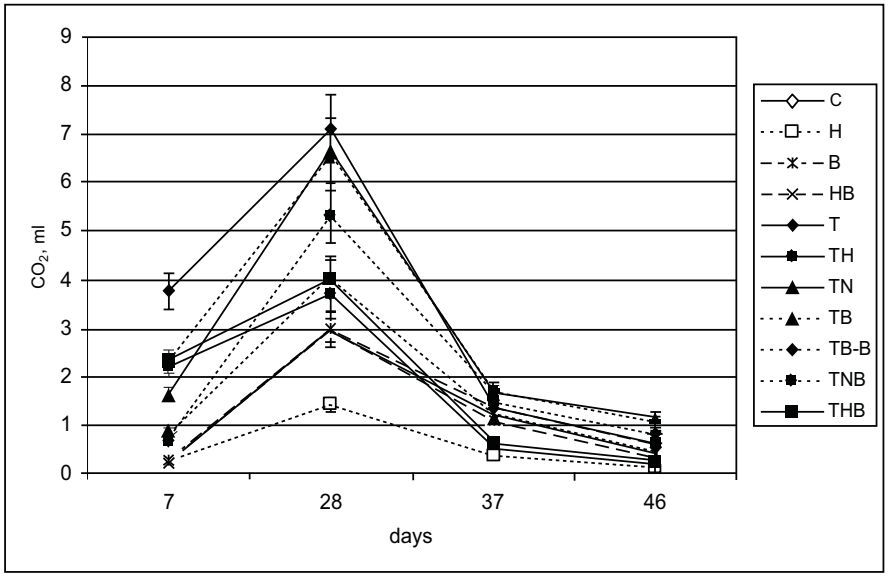

Fig. (2). Carbon dioxide production during fermentation of untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum, C. paraputrificum, Enterobacter asburiae, E. cloacae, Tissierella praeacuta* and *Bacillus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

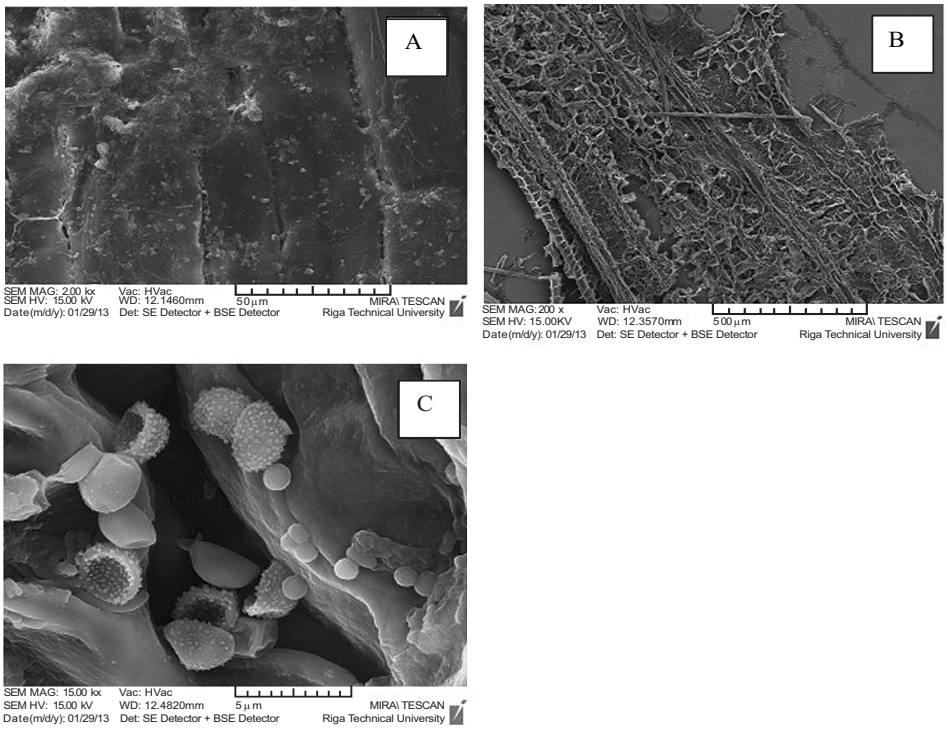

Fig. (3). SEM image of maize silage without pretreatment (A), pretreated with *Trichoderma asperellum* during three weeks (B), and pretreated silage after fermentation, with visible warty conidia of *T. asperellum* (C).

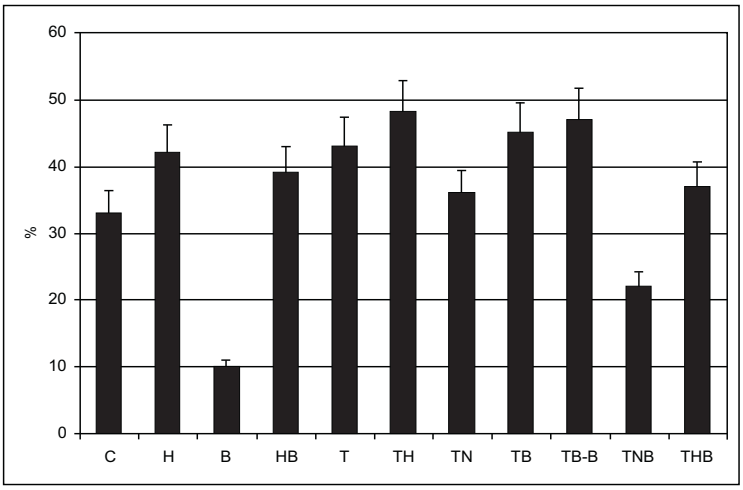

Fig. (4). Proportion of consumed amount of dry mass of silage during fermentation in comparison with initial input. Untreated (C) or with *T. asperellum* (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): *Clostridium butyricum, C. paraputrificum, Enterobacter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

was in the range from 20.8% to 30.6% and from 33.4% to 40.4% respectively, in variants with untreated silage and from 0.0% to 15.7% and from 2.8% to 42.4% respectively, in variants with pretreated silage. Butyric acid was the predominant acid in variant H with a proportion of 42.3% and in all pretreated variants with exception of TB. Moderate negative correlation r =-0.7138 (p =0.0136) was found between concentration of total volatile fatty acids (Table **3**) and pH (Table **2**). Moderate positive correlation r =0.7116 (p =0.0141) was found between concentration of total volatile fatty acids (Table **3**) and COD (Fig. **5**). Volatile fatty acids constituted from 30.1% (variant TNB) to 83.7% (variant T) from the COD remaining in the liquid after fermentation.

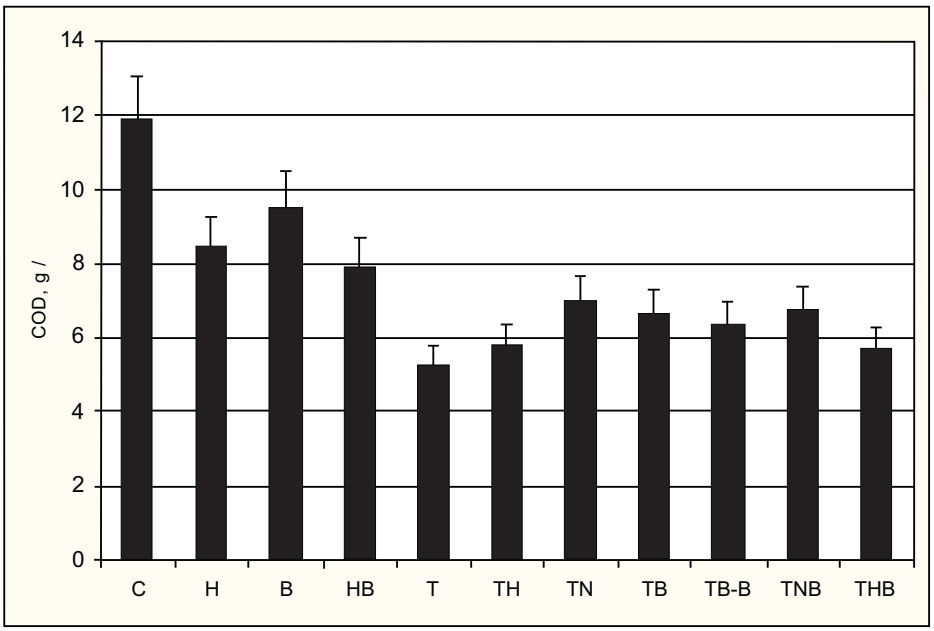

Fig. (5). Remaining COD (g/l) in solution after fermentation. Untreated (C) or with T. asperellum (T) pretreated silage supplemented with humic acids (H), ammonium nitrate (N), six bacteria (B): Clostridium butyricum, C. paraputrificum, Enterobacter asburiae, E. cloacae, Tissierella praeacuta and Bacillus mycoides, or five bacteria (B-B): Clostridium butyricum, C. paraputrificum, Enterobacter asburiae, E. cloacae and Tissierella praeacuta.

Table 3. Concentration of organic acids at the end of fermentation in mg/l (standard deviation did not exceed 10%) and proportion from total volatile fatty acids. 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*.

Variants	Formic acid		Acetic acid		Lactic acid		Propionic acid		Butyric acid		Total volatile fatty acids
	mg/l	%	mg/l	%	mg/l	%	mg/l	%	mg/l	%	mg/l
C	353	5.2	11.5	0.2	1791	26.2	2765	40.4	1916	28.0	6836.5
H	152	3.3	7.2	0.2	948	20.8	1523	33.4	1925	42.3	4555.2
B	118	3.0	3.2	0.0	1047	26.6	1573	39.9	1198	30.4	3939.2
HB	172	3.7	11.3	0.2	1420	30.6	1761	38.0	1272	27.4	4636.3
T	188	4.3	1.2	0.0	239	5.4	1053	23.9	2930	66.4	4411.2
TH	90	3.1	1.0	0.0	450	15.7	1062	36.9	1272	44.2	2875.0
TN	242	5.8	n	0.0	n	0.0	115	2.8	3791	91.4	4148.0
TB	152	4.3	1.1	0.0	448	12.7	1496	42.4	1430	40.5	3527.1
TB-B	170	5.9	1.5	0.0	255	8.9	971	33.8	1475	51.3	2872.5
TNB	135	6.7	n	0.0	n	0.0	161	8.0	1724	85.3	2020.0
THB	145	3.7	2.3	0.0	507	13.0	1432	36.8	1808	46.4	3894.3

n – not found

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* clasters.

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

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