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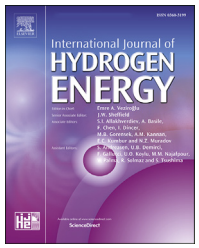

Inhibition of hydrogen-yielding dark fermentation by ascomycetous yeasts

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ABSTRACT

Hydrogen-yielding fermentation conducted in bioreactors is an alternative method of hydrogen production. However, unfavourable processes can seriously inhibit bio-hydrogen generation during the acidogenic step of anaerobic digestion. Here, ascomycetous yeasts were identified as a major factor inhibiting the production of bio-hydrogen by fermentation. Changes in the performance of hydrogen-producing bioreactors including metabolic shift, quantitative changes in the fermentation products, decreased pH, instability of the microbial community and consequently a dramatic drop in bio-hydrogen yield were observed following yeast infection. Ascomycetous yeasts from the genera *Candida*, *Kazachstania* and *Geotrichum* were isolated from hydrogen-producing bioreactors. Yeast metabolites secreted into the growth medium showed antibacterial activity. Our studies indicate that yeast infection of hydrogen-producing microbial communities is one of the serious obstacles to use dark fermentation as an alternative method of bio-hydrogen production. It also explains why studies on hydrogen fermentation are still limited to the laboratory or pilot-scale systems.

Introduction

Dark fermentation, which forms part of the acidogenic step of anaerobic digestion, is an alternative biological method of bio-hydrogen production. As an innovative technology based on microbial processes, modern biogas plants are being constructed in which the hydrogen- (acidogenesis) and methane-yielding (acetogenesis and methanogenesis) stages of anaerobic digestion are separated in order to generate these useful gases under controlled conditions. Two-step or even more complex multi-step anaerobic digestion increases energy recovery from biomass in comparison to a one-step process [1–5].

Theoretically, only one-third of the biomass can be converted to bio-hydrogen by the process of hydrogen-yielding fermentation. Maximum bio-hydrogen yield during *Clostridium*-type fermentation is 4 moles of hydrogen per mole of glucose when all of the substrate is converted to acetate according to the following equation: $C_6H_{12}O_6 + 2\,H_2O \rightarrow 4\,H_2 + 2\,CO_2 + 2\,CH_3COOH$. In practice, this value is lower due to the formation of non-gaseous products such as alcohols and organic acids other than acetate [6–9]. Several well-recognized

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factors can reduce bio-hydrogen production: (i) an increase in hydrogen partial pressure that inhibits the activity of NADH:-ferredoxin oxidoreductase (NFOR), an enzyme that catalyzes the formation of reduced ferredoxin in the reaction with NADH according to the equation NADH + Fd → NAD^+ + FdH; (ii) metabolic shift – the accumulation of acids causing a drop in pH in the bioreactor, which is unfavourable for bio-hydrogen production (the optimal pH for this process is around 5); (iii) coexistence of hydrogen-consuming microorganisms such as methanogens and acetogens; (iv) substrate competition between microorganisms including the replacement of hydrogen fermentation by lactic acid or ethanol fermentation; (v) excretion of antibacterial factors such as bacteriocins by lactic acid bacteria (LAB), inhibiting the growth of other bacteria; (vi) various toxic or inhibitory compounds [9–12].

Here, the presence of ascomycetous yeasts in the hydrogen-producing microbial community is shown as another factor that can seriously inhibit bio-hydrogen production during acidogenesis, while having no influence on the methane-yielding steps of anaerobic digestion. This inhibitory effect appears to depend on substrate competition and the production of antibacterial compounds (aforementioned factors iv and v). The negative impact of yeast infection on hydrogen-yielding bioreactors was suspected but so far there have been no reports confirming this.

Many ascomycetous yeast strains produce toxins that inhibit the growth of other yeasts, some fungi and bacteria. However, previous studies have focused mainly on their "killer" activity against yeasts and fungi rather than bacteria. The antimicrobial activities of yeast toxins are usually considered in positive terms: as a desirable factor preventing spoilage of fermented foods or beverages, or as probiotic and antidiarrheal factors used in medical or veterinary applications [13–15]. The "killer" yeasts belong to the genera *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula, Kluyveromyces, Metschnikowia, Pichia, Saccharomyces, Torulopsis, Ustilago, Williopsis* and *Zygosaccharomyces*. "Killer" toxins are generally proteins or glycoproteins of low molecular weight (<20 kDa) that are encoded by chromosomal genes (e.g. KHR and KHS of *Saccharomyces cerevisiae*; HM-1 and K-500 of *Williopsis mrakii*), genes carried by linear double-stranded DNA plasmids (e.g. pGkL1 and pGkL2 of *Kluyveromyces lactis*; pPac 1-1, pPac 1-2 of *Pichia acacia*) or by cytoplasmically-inherited encapsulated double-stranded RNA viruses (e.g. K1, K2 and K28 of *Saccharomyces cerevisiae*; P1, P4 and P6 of *Ustilago maydis*). The mechanisms of "killer" toxins active against yeasts are relatively well characterized and include blocking DNA synthesis, inhibiting synthesis of the cell wall component β-1,3-glucan and disrupting the cell membrane by forming channels in the lipid bilayer [13,14,16]. Several antagonistic properties of yeasts against bacteria have been identified. These include competing for nutrients, causing pH changes, producing high concentrations of ethanol, and inhibiting bacterial attachment to intestinal cells [14].

We have previously described the development and testing of two-stage anaerobic digestion systems of different scales: a laboratory scale system and a pilot scale installation. These generate bio-hydrogen (in stage 1) and methane (in stage 2) under mesophilic conditions using sucrose-rich by-products [17–19], as well as intermediate and waste products, of the sugar beet refining industry as the primary energy substrate. In this report, it is shown how the performance of hydrogen-producing bioreactors is adversely affected by infection with ascomycetous yeasts introduced either in the fermentation substrate or as an external contaminant. Tests for growth inhibition confirmed the inhibitory activity of yeasts obtained from the bioreactors against bacterial indicator strains and a sample of hydrogen-yielding microbial community. In addition, proteins present in a crude filtrate of a *Candida humilis* culture were characterized by mass spectrometric analysis.

Materials and methods

Source and identification of ascomycetous yeast isolates

Previously, a pilot scale installation (30–50-L bioreactors) producing bio-hydrogen and methane from molasses (a by-product of the sugar industry) in a two-stage anaerobic digestion performed under mesophilic conditions was described. The installation currently operates in a Polish sugar factory [17]. In this system, bio-hydrogen is generated via acidogenic processes in a packed bed reactor (PBR) by a hydrogen-yielding microbial community fermenting molasses. The non-gaseous organic products from this stage then feed an upflow anaerobic sludge blanket (UASB) reactor in which methane (biogas) is produced by a methane-yielding microbial community. The yeast strains with antibacterial activity that are the subject of this study were isolated from the hydrogen-producing bioreactor. Samples from this bioreactor were plated on solid YPD medium (10 g/L bacto yeast extract, 20 g/L bacto-peptone, 20 g/L dextrose, 20 g/L agar; [20]) and incubated aerobically at room temperature (22–24 °C). Eleven yeast colonies were picked. These were Gram-stained and examined using a light microscope (Nikon Eclipse E200; 100× objective lens).

Genomic DNA was isolated from the yeasts using a PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's protocol up to the DNA precipitation step. The yeast DNA was precipitated with 0.6 vol of isopropanol at room temperature (22–24 °C) for 30 min, then collected by centrifugation at 14,000 × *g* for 20 min. The pellets were washed with cold 70% ethanol, dried and resuspended in 100 μl sterile deionized water. Approximately 350 ng of purified DNA was used as the template for PCR amplification (using a Bio-Rad T100 Thermal Cycler) of hypervariable region ITS1 and a fragment of the 28S rRNA gene using the primer pairs ITS1 (5′ CTT GGT CAT TTA GAG GAA GTA A 3′)/ITS2 (5′ GCT GCG TTC TTC ATC GAT GC 3′), ITS1/LR5 (5′ TCC TGA GGG AAA CTT CG 3′) and NL1 (5′ GCA TAT CAA TAA GCG GAG GAA AAG 3′)/NL4 (5′ GGT CCG TGT TTC AAG ACG G 3′) [21]. For the primer pair ITS1/ITS2 the following amplification conditions were used: 95 °C for 10 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 90 s, with a final extension at 72 °C for 10 min. For the ITS1/LR5 and NL1/NL4 primers pairs the following amplification conditions were used: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products were directly sequenced using BigDye Terminator v.3.1 chemistry on an ABI3730xl DNA Genetic Analyzer (Applied Biosystems) with the primers used for the

PCR. The sequencing results in the form of ab1 files were assembled with Geneious assembler software using default parameters, except that the Error Probability Limit was set to 0.005 (Geneious 10.2.3; http://www.geneious.com; [22]). The assembled sequences were used as queries in Megablast searches [23] against the nr NCBI database.

Growth inhibition test

Microorganisms used for the growth inhibition test (Table 1) were pure cultures of various indicator bacteria, a sample of hydrogen-producing microbial community characterized previously [18], a laboratory strain of *Pichia pastoris* SMD1169 and the yeast isolates described in the previous section.

The strains of *Klebsiella oxytoca* and *Citrobacter freundii* used were isolated from a hydrogen-producing bioreactor described previously [18]. Briefly, samples from the bioreactor were plated on M9 medium [24] containing molasses (40 g/L) and solidified with 2% agar (Difco) then incubated in an anaerobic chamber (Vinyl Anaerobic Chamber Coy Laboratory Products, Inc.) at room temperature (22–24 °C). Bacterial colonies were picked and propagated, then genomic DNA was extracted using a Genomic Mini isolation kit (A&A Biotechnology) according to the manufacturer's instructions. Approximately 100 ng of DNA was used as the template for PCR amplification of nearly full-length bacterial 16S rRNA gene fragments using MARATHON polymerase (A&A Biotechnology) with the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The reactions were performed in a PTC-200 thermal cycler (MJ Research, Inc., USA) using the following conditions: 95 °C for 5 min, followed by 20 cycles of 95 °C for 30 s, 53 °C for 30 s, 72 °C for 90 s; and then 5 cycles of 95 °C for 30 s, 46 °C for 30 s, 72 °C for 1.5 min; with a final extension at 72 °C for 10 min. Amplification products purified with a NucleoSpin Extract II kit (Macherey-Nagel) were directly sequenced using BigDye Terminator v.3.1 chemistry on an ABI3730xl DNA Genetic Analyzer (Applied Biosystems) with the primers F27, 1492R, F357 (5′-GCCTACGGAGGCAGCAG-3′), 519R (5′-ATTACCGCGGCTGCTGG-3′), and 926R (5′-CCGTCAATTCCTTTGAGTTT-3′). The DNA sequences were assembled using the Linux-based software phred/phrap/consed and checked manually. The obtained 16S rDNA sequences were then used as queries against the NCBI database using BLAST. The two isolates displayed 100% similarity to *Klebsiella oxytoca* strains 5 and 8 and *Citrobacter freundii* YRL11, respectively.

Strains of *Leuconostoc mesenteroides*, *Lactobacillus casei*, *L. paracasei* and *L. plantarum* were isolated from milk and dairy products [25].

The specific growth media used for the cultivation of the different microorganisms are shown in Table 1: Luria–Bertani

Table 1 – Microorganisms and growth media used in growth inhibition tests.

Microorganism	References	Growth medium	Growth conditions
Yeasts			
Candida humilis isolates	This work	acidic YPD, pH 4.5	Aerobic/
Kazachstania exiqua isolate	This work	neutral YPD, pH 7	Anaerobic
Geotrichum candidum isolate	This work		
Pichia pastoris SMD1169	Invitrogen		
Bacteria			
Escherichia coli K12 AB1157	[47]	LB, pH 6.9	Aerobic
Pseudomonas putida PaW85 isogenic to KT2440	[48,49]	LB + neutral YPD (1:1), pH 6.8 LB + acidic YPD (1:1), pH 5.0	
Citrobacter freundii isolate	Selected and identified in 2010 from a hydrogen-producing microbial community described previously [18], see the text	LB + neutral YPD (1:5), pH 6.7 LB + acidic YPD (1:5), pH 6.6	
Klebsiella oxytoca isolate	As *Citrobacter freundii* isolate		
Bacillus megaterium MS941	MoBiTec, BMEG50		
Clostridium butyricum 2478	DSMZ collection, DSM-2478	CDA, pH 7	Anaerobic
Hydrogen-producing microbial community	[18]	CDA + neutral YPD (1:1), pH 6.9 CDA + acidic YPD (1:1), pH 5.3 CDA + neutral YPD (1:5), pH 6.8 CDA + acidic YPD (1:5), pH 6.5	
Leuconostoc mesenteroides subs. *mesenteroides*	A kind gift from prof. Jacek Bardowski [25]	MRS, pH 5.5 MRS + neutral YPD (1:1), pH 6.2 MRS + acidic YPD (1:1), pH 5.2 MRS + neutral YPD (1:5), pH 5.6 MRS + acidic YPD (1:5), pH 5.5	
L. mesenteroides subs. *dextranicum*			
L. mesenteroides subs. *dextranicum* 3158			
Lactobacillus paracasei 3114			
L. paracasei 3130			
Lactobacillus plantarum 3123			
L. plantarum 3078			
L. plantarum 3107			
Lactobacillus casei 3084			
L. casei 3129			
L. casei 3035			

broth (LB) [24]; clostridial differential medium (CDA) (Sigma Aldrich); MRS medium (Merck). *E. coli*, *C. freundii*, *K. oxytoca*, *P. putida*, *S. oneidensis* and *B. megaterium* were grown aerobically in LB medium at 30 °C with shaking overnight. *C. butyricum*, LAB and the H_2-producing microbial community were grown anaerobically in a Vinyl Anaerobic Chamber (Coy Laboratory Products, Inc.) or a stainless steel anaerobic jar (Schuett-Biotec, Germany) in the media described in Table 1 without shaking at 30 °C for 72 h. Yeasts were grown aerobically or anaerobically (in an anaerobic jar) in YPD medium (pH 4.5 or 7.0) at room temperature (22–24 °C) without shaking for 72 h and then the cultures were filtered through a 0.2-μm PES membrane using a VWR Vacuum Filtration System. The YPD medium was buffered to pH 4.5 using 0.1 M citrate-phosphate buffer [26]. The protein concentration of the crude filtrate, measured by Bradford's method [27], was 0.045 μg/μl.

The crude yeast culture filtrate was mixed with the required bacterial growth medium in a 1:1 or 5:1 ratio to give a total volume of 6 ml, and then inoculated with 50 μl of overnight cultures of the respective indicator bacteria. The control samples contained YPD (pH 4.5 or 7.0) instead of the crude filtrate. The pH of the media is given in Table 1. The bacteria were then grown aerobically or anaerobically (anaerobic chamber or anaerobic jar) at room temperature (22–24 °C) without shaking for 24 h, and then the OD_{600} of the cultures was measured. Inhibition of bacterial growth by the crude filtrate was calculated according to the following formula: 100% × (OD_{600} control − OD_{600} bacteria grown in the presence of the filtrate)/OD_{600} control.

Analytical methods

Analytical methods used to characterize the performance of the hydrogen-producing bioreactor were as described previously [17]. Briefly, the total rate of hydrogen-rich fermentation gas production was measured using a bubble flowmeter (Zakłady Urządzeń Przemysłowych ZAM Kęty, Poland) and its composition was analyzed by gas chromatography with a thermal conductivity detector (GC/TCD) using an Agilent Technologies model 7890B gas chromatograph.

The pH and chemical oxygen demand (COD) of the acidic effluent from molasses fermentation were measured using a standard pH meter (ELMETRON model CP-502, Poland) and a NANOCOLOR COD 1500 kit (Machery-Nagel; according to ISO 1575:2002), respectively. The concentration of carbohydrates in the acidic effluent from molasses fermentation and in the molasses-containing medium were analyzed using high performance liquid chromatography (HPLC) with refractometric detection (Waters HPLC system: Waters 2695 - Separations Module, Waters 2414 - Refractive Index Detector, and 300 × 6.5 mm Sugar Pak column with guard column). Short-chain fatty acids were analyzed by HPLC with photometric detection (Waters HPLC system as above, Waters 2996 – Photodiode ArrayDetector, and 300 × 7.8 mm Aminex HPX-87 H

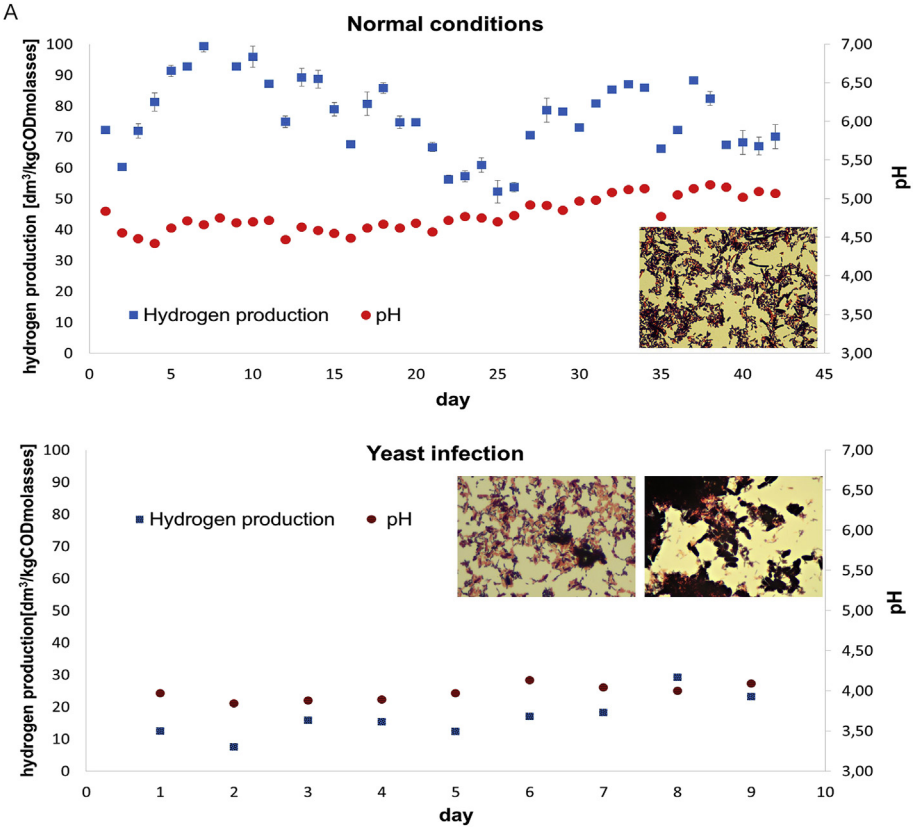


Fig. 1 – Effects of yeast infection on hydrogen-yielding microbial communities: bio-hydrogen production and pH of the effluent, n = 10 for each point, including microscopic images (A); percentage contribution of bio-hydrogen in fermentation gas (B); non-gaseous products of fermentation, n = 5 (C). In each case the mean ± SD (standard deviation) was calculated.

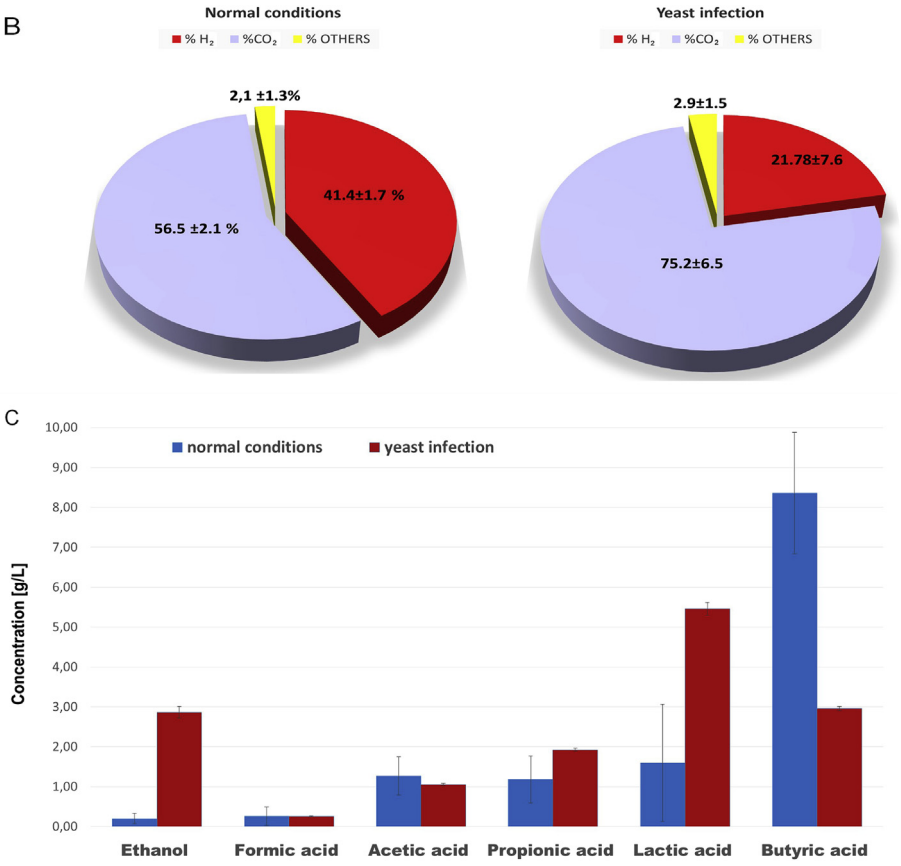


Fig. 1 – (*continued*).

column with guard column). Ethanol was quantified by gas chromatography with flame-ionization detection (Hewlett Packard 6890, autosampler headspace - Hewlett Packard 7694E, polar 1.0-μm capillary column and FID). The HPLC conditions for these analyses were as described previously [18,19].

Samples taken from the bioreactor were Gram-stained and examined using a light microscope (Nikon Eclipse E200; 100× objective lens).

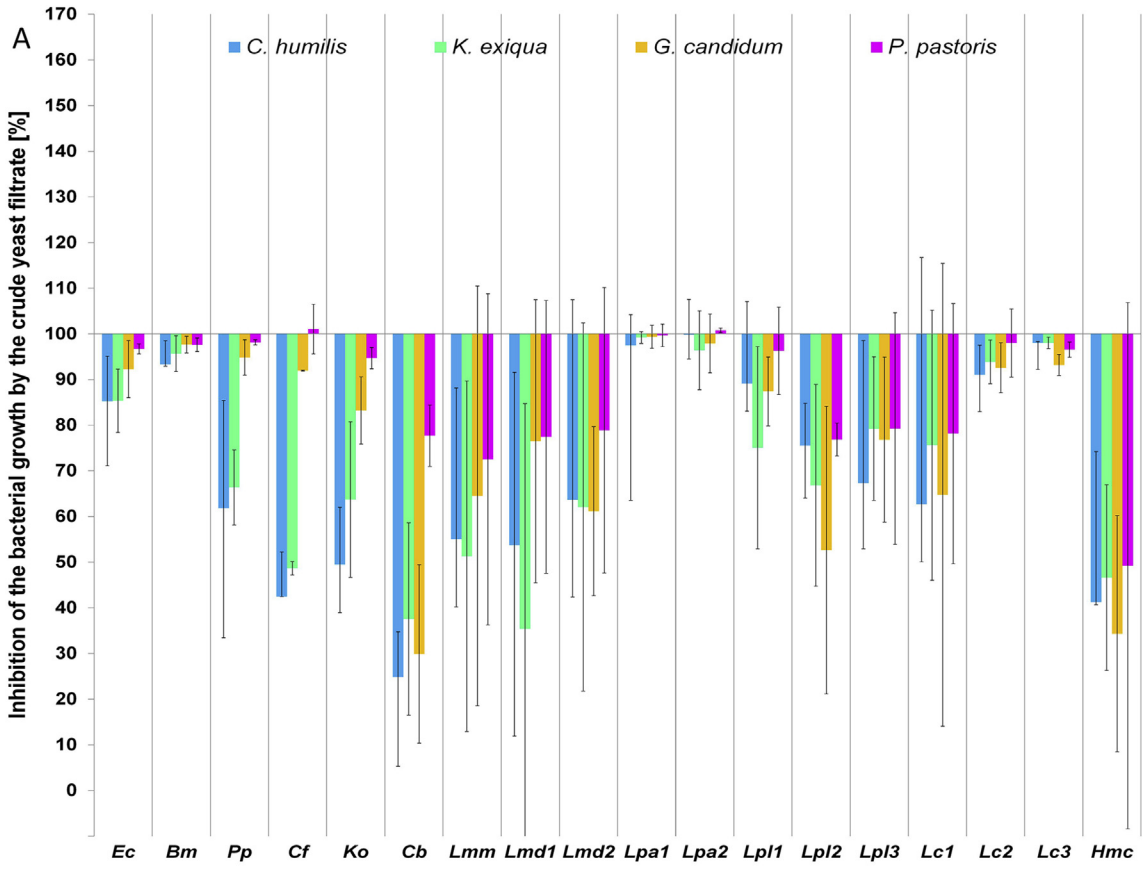
Isolation of proteins secreted by Candida humilis *and use in growth inhibition tests*

A 1-L culture of *Candida humilis* (isolate 1) was grown in acidic YPD medium (Table 1) under aerobic conditions at 30 °C for 3 days. This was filtered through a 0.22-μm filter, dialyzed against buffer A (20 mM Tris-HCl pH 8.0, 10 mM NaCl) and concentrated to 15 ml by Tangential Flow Filtration (TFF) and by ultrafiltration using a 5-kDa cutoff Pellicon XL Ultrafiltration Module Biomax (Merck Millipore). The concentrated filtrate was subjected to anion exchange chromatography on a HiTrap Q XL column (GE Healthcare) with a linear gradient from 100% buffer A to 100% buffer B (20 mM Tris-HCl pH 8.0, 1 M NaCl). Four peaks were obtained (Supplementary Fig. S1). The filtrate fractions corresponding to these peaks were concentrated using Amicon Ultra-4 centrifugal filter units with a 5-kDa cut-off (Merck Millipore). Simultaneously, the YPD medium was concentrated and separated by anion exchange chromatography in a similar way. This produced 5 distinguishable peaks (Supplementary Fig. S2). All culture filtrate and medium fractions were analyzed by electrophoresis on stain-free, 4–15% gradient SDS-PAGE gels (Bio-Rad) (Supplementary Fig. S3). Any distinct bands were excised from the gels and the constituent proteins purified. The protein concentration of the filtrate fractions (I–IV), measured by Bradford's method [27], were as follows: I – 0.0 μg/μl, II – 0.64 μg/μl, III – 1.41 μg/μl, IV – 0.48 μg/μl.

A sample of the concentrated filtrate and the two visible bands MS1 and MS2 isolated from filtrate fractions II and IV, respectively, were analyzed by MS. Protein identities and functions were retrieved from the UniProt database [28].

Mass spectrometric analysis of protein fractions

MS analysis was performed in the Mass Spectrometry Laboratory at the Institute of Biochemistry and Biophysics PAS according to standard procedures. A sample of the concentrated filtrate was suspended in 100 mM ammonium bicarbonate (ABC). The proteins were then reduced with 0.5 M (5 mM f.c.) Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 1 h at 60 °C, blocked with 200 mM S-Methyl methanethiosulfonate (MMTS) (10mM f.c.) for 10 min at RT and digested overnight with 10 ng/μl trypsin (sequencing Grade Modified Trypsin, Promega V5111). Proteins recovered from the gel bands MS1 and MS2 from filtrate fractions II and IV, respectively, were reduced with 100 mM 1,4-Dithiothreitol (DTT) for 30 min at 57 °C, alkylated with 0.5 M iodoacetamide for 45 min in darkness at room temperature and then


A
C. humilis
K. exiqua
G. candidum
P. pastoris
Inhibition of the bacterial growth by the crude yeast filtrate [%]
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Ec
Bm
Pp
Cf
Ko
Cb
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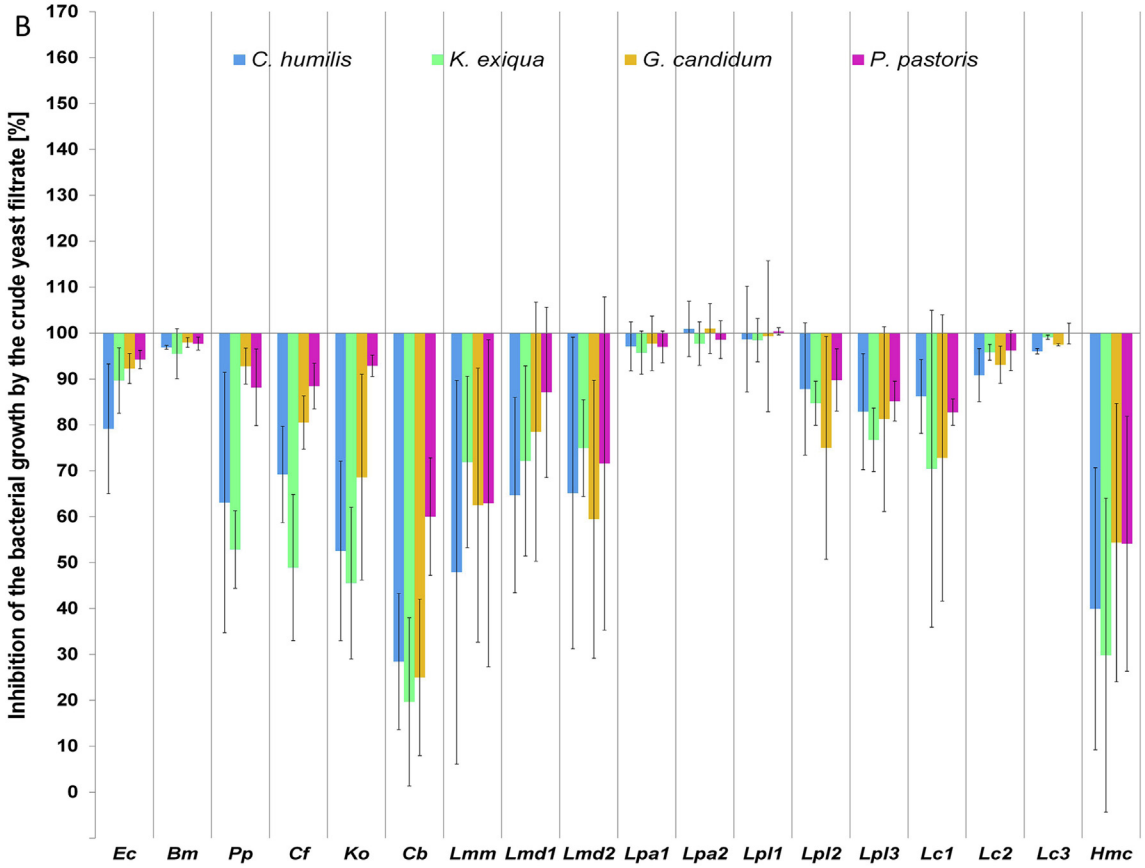
Lpl2
Lpl3
Lc1
Lc2
Lc3
Hmc


B
C. humilis
K. exiqua
G. candidum
P. pastoris
Inhibition of the bacterial growth by the crude yeast filtrate [%]
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Ec
Bm
Pp
Cf
Ko
Cb
Lmm
Lmd1
Lmd2
Lpa1
Lpa2
Lpl1
Lpl2
Lpl3
Lc1
Lc2
Lc3
Hmc

suspended in 25mM ABC and digested overnight at 37 °C with 10 ng/μl of trypsin added directly to the reaction mixture. Peptides were eluted with 2% acetonitrile in the presence of 0.1% TFA. The resulting peptide mixtures were applied to a RP-18 pre-column (Waters, Milford, MA) using water containing 0.1% FA as the mobile phase and then transferred to a nano-HPLC RP-18 column (internal diameter 75 μM, Waters, Milford MA) using an acetonitrile (ACN) gradient (0–35% ACN in 160 min) in 0.1% FA at a flow rate of 250 nL/min. The column outlet was coupled directly to the ion source of an Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA) working in the regime of data-dependent MS to MS/MS switch. To ensure the absence of cross-contamination from previous samples a blank run preceded each analytical run. The acquired MS/MS data were pre-processed with Mascot Distiller software (MatrixScience, London, UK) and a search was performed against the Uniprot database of annotated *Kazachstania* species proteins (download date 2017.07.19, 159566 sequences) using the Mascot Search Engine (MatrixScience, London, UK, Mascot Server). The following Mascot search parameters were employed: mass tolerance for peptides – 30 ppm, mass tolerance for fragments – 0.1 Da, enzyme – trypsin; missed cleavages – 1; variable modifications – oxidation (M), carbamidomethyl (C), methylthio (C), carbamidomethyl (N-term); instrument – HCD. Peptides were accepted on the basis of the Mascot-derived score threshold. The obtained masses were used in searches of the NCBIprot database restricted to the genera *Candida*, *Kazachstania*, and *Saccharomyces*, in Mascot server. The search results for the concentrated filtrate and for the bands MS1 and MS2 from filtrate fractions II and IV (Supplementary Fig. S3) are available as Supplementary Table S1.

The YPD medium was not examined by MS analysis because no distinguishable protein bands were detected by SDS-PAGE analysis and this medium did not inhibit bacterial growth.

Results and discussion

Performance of hydrogen-yielding bioreactors under normal conditions and after yeast infection

Previously, conditions for continuous hydrogen-rich fermentation gas production from molasses in laboratory scale (3-L) and pilot scale (30-L) packed bed bioreactors were established [17,18]. In these systems the microbial community formed granules and biofilms, and the granular sludge promoted bio-hydrogen production. Microscopic observations revealed various morphological forms of Gram-positive and Gram-negative bacteria within this sludge. The fermentation gas generated, contained around 40% (v/v) bio-hydrogen and the 30-L bioreactor was able to produce up to 60 dm^3 H_2/kg COD molasses.

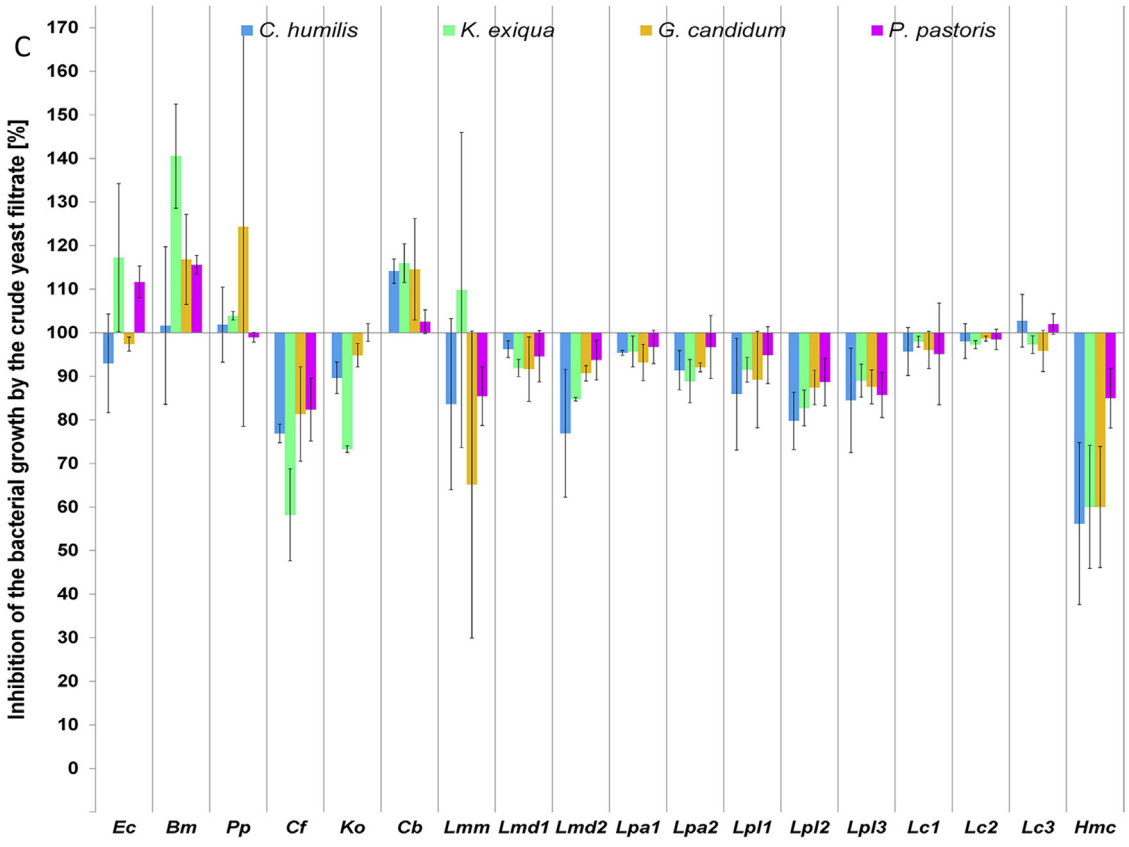
Conditions in the bioreactors were indicated by the pH of the effluent. When this value was maintained at around 5, conditions were favourable for bio-hydrogen production. The pH was determined by the levels of the non-gaseous fermentation products and the value of 5 indicated the prevalence of butyrate. Other fermentation products detected in the acidic effluent were formic, acetic, lactic and propionic acids as well as ethanol.

Dramatic changes in the performance of the hydrogen-producing bioreactor were observed when the microbial community was infected by yeasts. Fig. 1 shows various parameters of a 30-L hydrogen-producing bioreactor packed with slag during selected periods of its activity under normal conditions and after yeast infection. Fig. 1 shows representatives data describing the hydrogen-producing cultures in the periods of yeast infection.

Microscopic analysis (Fig. 1A) of the hydrogen-yielding microbial community showed a change in the bacterial component after yeast infection, with an increased tendency for the cells to agglutinate. Yeast infection also produced the following changes in the function of the bioreactor: (i) a 2–3-fold decrease in the total rate of gas production and bio-hydrogen content in the fermentation gas (Fig. 1A and B); (ii) a drop in pH of the acidic effluent from 4.5–5 to < 4.0 (Fig. 1A); (iii) an increase in the concentration of ethanol (14-fold) and lactate (3-fold), and a decrease in butyrate (3-fold) in the acidic effluent (Fig. 1C).

These data clearly show that the presence of yeast in the hydrogen-yielding microbial community seriously inhibit bio-hydrogen production during acidogenesis due to unfavourable changes in fermentation processes. These yeasts compete with bacteria for substrate and are the reason for the partial replacement of hydrogen fermentation by ethanol fermentation. Furthermore, the yeasts secrete antibacterial factors into the bioreactor environment. In combination these phenomena trigger a cascade of processes that are unfavourable for bio-hydrogen production: a decrease in the concentration of

Fig. 2 – Inhibition of bacterial growth by secreted products present in crude yeast culture filtrates. Yeast strains isolated from the hydrogen-yielding fermentation bioreactor (*C. humilis*, *K. exiqua*, *G. candidum*) and *P. pastoris* SMD1169 were cultured in YPD medium. Crude filtrates were prepared from cultures grown (A) anaerobically at pH 4.5 and diluted in the respective bacterial media in the ratio 1:1, (B) aerobically at pH 4.5 and diluted in the respective bacterial media in the ratio 1:1, (C) aerobically at pH 4.5 and diluted in the respective bacterial media in the ratio 1:5, (D) aerobically at pH 7.0 and diluted in the respective bacterial media in the ratio 1:1, before inoculation with a panel of indicator bacteria. The bacteria were cultured aerobically or anaerobically at room temperature for 24 h and the level of growth inhibition was calculated from OD_{600} measurements. The results come from 3 to 5 experiments. In each case a mean ± SD (standard deviation) was calculated. *Ec* – *E. coli* K12 AB1157; *Pp* – *P. putida* PaW85; *Bm* – *B. megaterium* MS941; *Cf* – *C. freundii*; *Ko* – *K. oxytoca*; *Cb* – *C. butyricum* 2478; *Lmm* – *L. mesenteroides* subs. *mesenteroides*; *Lmd1* - *L. mesenteroides* subs. *dextranicum*; *Lmd2* - *L. mesenteroides* subs. *dextranicum* 3158; *Lpa1* – *L. paracasei* 3114; *Lpa2* – *L. paracasei* 3130; *Lpl1* – *L. plantarum* 3123; *Lpl2* – *L. plantarum* 3078; *Lpl3* – *L. plantarum* 3107; *Lca1* – *L. casei* 3084; *Lca2* – *L. casei* 3129; *Lca3* – *L. casei* 3035; *Hmc* - Hydrogen-producing microbial community.



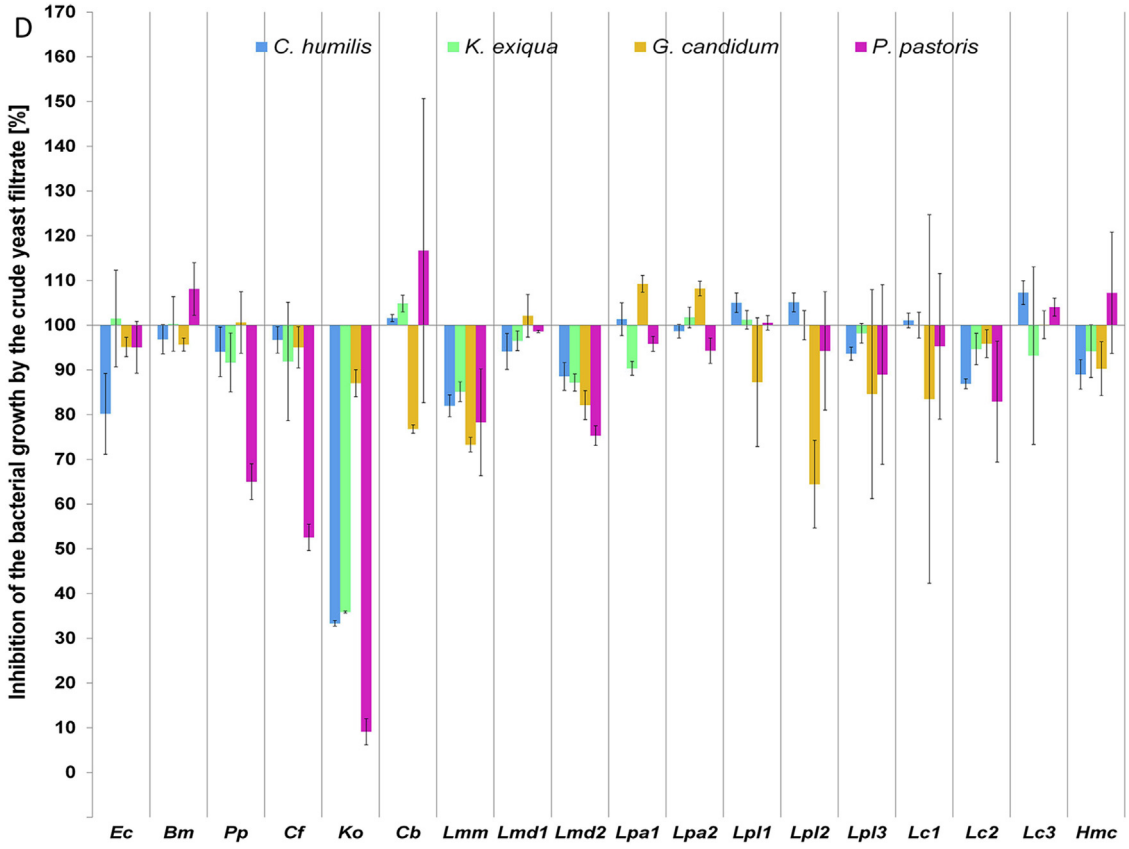


Fig. 2 – (continued).

butyric acids and increases in the concentrations of ethanol and lactic acid, leading to a fall in pH and changes in the structure and balance of the microbial community.

Butyrate is recognized as the predominant metabolite during butyric acid fermentation at pH 5.0–5.5 [29]. It has also been proposed that a phenomenon analogous to cross-feeding observed within the gastrointestinal tract occurs in hydrogen-producing bioreactors [3,18,30]. Cross-feeding in intestinal bacteria involves the conversion of lactate and acetate to butyrate and bio-hydrogen by butyrate-producing bacteria, stimulated by LAB [30,31]. In many studies on microbial bio-hydrogen production, the pH was found to be the critical factor for bio-hydrogen production from lactate and acetate [32–34]. The pH seems to be crucial for establishment of the correct balance between butyrate-producing and LAB, and any changes in this parameter disturb the specific "homeostasis" of hydrogen-producing microbial communities in bioreactors.

Unfortunately, an acidic environment (pH 4–5) is optimal for the growth of many ascomycetous yeasts and favours their "killer" activity. Many of the proteins necessary for "killer" activity mature, are stabilized and attain maximal activity in environments with pH 3–5 and mesophilic conditions. This enhanced activity is then maintained even after a subsequent shift to a neutral environment [35,36].

The presence of yeast in the hydrogen-yielding microbial community severely inhibited bio-hydrogen production during acidogenesis, but had no effect on the methane-yielding steps of anaerobic digestion. The methane yield in the second step of the described system was comparable to that found previously [17]. A similar amount of methane was produced regardless of any yeast infection affecting the first step (data not shown). This insensitivity to the presence of yeast contaminants in the microbial community is one of the main reasons why methane, as the end product of anaerobic digestion of biomass, is widely used as a source of energy, whereas the exploitation of more sensitive hydrogen-yielding fermentations is still in its infancy. The neutral/slightly alkaline environment in methane-yielding bioreactors may be the main reason why "killer" yeasts do not disrupt the methane-producing microbial communities.

The primary source of the "killer" yeasts that disturb bio-hydrogen production was sugar beet waste products. These yeasts thrived in the environment of the hydrogen-producing bioreactor. Efficient methods of biomass pre-treatment are required to prevent yeast growth in hydrogen-yielding microbial communities. This will need to be addressed if sucrose-rich by-products, bio-wastes and intermediate products of the sugar industry are to be used for the production of gas biofuels, i.e. bio-hydrogen and bio-methane (biogas).

Identification of the yeast isolates

Cultivable yeasts were isolated from the hydrogen-yielding bioreactor during three periods of yeast infection using classical microbiological techniques. Eleven yeast isolates were obtained and identified by analysis of their hypervariable region ITS1 and 28S rRNA gene fragment sequences amplified by PCR from purified genomic DNA. Nine of the isolates were most closely related to *Candida humilis* strain UWO(PS) 92–219.1, one to *Kazachstania exiqua* strain WY3, and one to *Geotrichum candidum*. These yeasts were isolated from sour-dough cultures, kefir cultures, dairy products and the gastro-intestinal tract [15,37–40].

Inhibition of bacterial growth by crude yeast culture filtrates

The inhibitory effect of factors secreted by yeasts was tested in assays using a panel of indicator bacteria. Crude culture filtrates were obtained after 72-h growth of the isolated yeast strains (*C. humilis* isolate 1, *K. exiqua*, *G. candidum*) and *P. pastoris* SMD1169 in YPD medium. The crude filtrates were prepared from cultures grown anaerobically and aerobically at pH 4.5 or 7.0, and were diluted in the respective bacterial growth media at ratios of 1:1 or 1:5 before inoculation with the bacterial strains. The pH values of the obtained media mixtures are shown in Table 1. Dilution of the acidic YPD in LB or CDA bacterial media at the ratio of 1:5 resulted in an increase in pH to 6.5–6.7. Bacterial growth inhibition caused by treatment with the different culture filtrates is shown in Table 1.

The inhibitory effect of the secreted yeast products was greatest for bacteria grown in the acidic environment (pH 4.5) when the ratio of crude filtrate to medium was 1:1, irrespective of whether the examined yeasts were cultivated aerobically or anaerobically (Fig. 2). The greater antibacterial effect occurring when the yeasts were grown at pH 4.5, in comparison to neutral conditions (pH 7.0), was due to the acidic conditions (pH 4–5) which are optimal for "killer" activity (as mentioned above). Tests of the sensitivity of bacterial strains to "killer" yeasts used in previous studies were mainly performed in acidic environments [26,41,42].

A particularly interesting group among the tested bacteria are LAB. Their sensitivity to products secreted by "killer" yeasts is dependent on the isolate rather than the species. This is especially evident among members of the genus *Lactobacillus* (Fig. 2A and B). In experiments using crude filtrates from yeast cultures grown in neutral medium or when the ratio of the filtrate to the medium was 1:5, no differences in the sensitivity of LAB were observed (Fig. 2C and D). In the study of Meneghin and co-workers (2010) [42], *B. subtilis*, *L. plantarum*, *L. fermentum* and *L. mesenteroides* showed different sensitivities to "killer" yeasts from the genera *Saccharomyces*, *Candida*, *Pichia*, *Trichosporon* and *Kluyveromyces*. However, in contrast to these results, Izgu and Altinbay (1997) [26] found that "killer" toxins of *Hansenula*, *Candida*, and *Kluyveromyces* species only had an inhibitory effect on the growth of Gram-positive bacteria such as *Bacillus subtilis*, *Sarcina lutea*, *Streptococcus pyogenes* and *Staphylococcus aureus*. Gram-negative *Proteus vulgaris*, *Shigella flexneri*, *Klebsiella pneumonia*, *Salmonella typhimurium*, *E. coli* and *P. aeruginosa* were resistant to "killer" yeasts. In agreement with the results of Meneghin and co-workers [42] both Gram-positive and Gram-negative bacteria were found as sensitive to the crude yeast culture filtrates.

LAB and yeasts are the predominant microbes involved in the fermentation of foods including cereals, vegetables and milk. A balance of microorganisms and interactions between particular groups ensure the desired profile of flavour compounds, produce the typical characteristics of the product and prevent spoilage. Compounds secreted by yeasts may enhance bacterial growth (e.g. by vitamin production) at neutral pH or inhibit it after acidification, depending on the

phase of the fermentation process [40,43]. This would explain the stimulation of growth of some bacteria observed in the presence of crude filtrates from yeast cultures grown in the neutral medium or when the ratio of filtrate to medium was 1:5. In the latter case, any potential toxin was diluted and the medium mixtures (YPD:LB or YPD:CDA) showed an increase in pH (Fig. 2C and D).

The presented results clearly show that secreted products of yeast metabolism inhibit bacterial growth. Further studies are necessary to elucidate the interaction between bacteria and yeasts in hydrogen-producing bioreactors.

Characterization of secreted yeast products in crude culture filtrates

To further investigate the nature of the potential antibacterial factor(s) secreted by yeasts isolated from the hydrogen-producing microbial community, the *C. humilis* isolate 1 was chosen. *C. humilis* was the yeast species most frequently isolated from the examined samples. Notably, the genera *Candida* and *Kazachstania* are closely related [44].

MS analysis of the concentrated culture filtrate identified 76 proteins with homology to database entries for the genera *Candida*, *Kazachstania* and *Saccharomyces*, 40 of which originated from *Candida* spp./*Kazachstania* spp. MS analysis of a band MS1 at approx. 50 kDa recovered from an SDS-PAGE gel after electrophoretic separation of fraction II identified 68 proteins including 43 originating from *Candida* spp./*Kazachstania* spp. MS analysis of a band MS2 at approx. 45 kDa band recovered after electrophoretic separation of fraction IV identified 40 proteins including 25 from *Candida* spp./*Kazachstania* spp.

An analysis of secreted and cell membrane proteins identified by MS analysis of the concentrated *C. humilis* culture filtrate and the gel-purified bands representing the separate fractions identified proteins involved in various cellular

Table 2 – Secreted (red) and cell membrane (blue) proteins identified by MS analysis of the concentrated *C. humilis* isolate 1 culture filtrate.

UniProt Accession no.	Score	Mass (Da)	Identity/closest homologue	GO- molecular function/ GO – biological process
G8BIX4	54	49,905	Putative uncharacterized protein OS=Candida parapsilosis (strain CDC 317 / ATCC MYA-4646) GN=CPAR2_403920 PE=4 SV=1	Integral component of membrane
J7S410	52	48,932	Uncharacterized protein OS=Kazachstania naganishii (strain ATCC MYA-139 / BCRC 22969 / CBS 8797 / CCRC 22969 / KCTC 17520 / NBRC 10181 / NCYC 3082) GN=KNAG0M00890 PE=3 SV=1	Glucan exo-1,3-beta-glucosidase activity; carbohydrate metabolic process; ascospore formation*
H0GWL1	50	31,099	Atg27p OS=Saccharomyces cerevisiae x Saccharomyces kudriavzevii (strain VIN7) GN=VIN7_7886 PE=4 SV=1	Integral component of membrane
B9WE14	45	46,994	Arginase, putative OS=Candida dubliniensis (strain CD36 / ATCC MYA-646 / CBS 7987 / NCPF 3949 / NRRL Y-17841) GN=Cd36_84150 PE=3 SV=1	Arginase, putative; arginase activity; metal ion binding
A0A1X7R8N1	43	142,368	Uncharacterized protein OS=Kazachstania saulgeensis GN=KASA_0J03179G PE=4 SV=1	Uncharacterized protein
G8B4Z5	41	50,172	Putative uncharacterized protein OS=Candida parapsilosis (strain CDC 317 / ATCC MYA-4646) GN=CPAR2_601050 PE=4 SV=1	Antiporter activity; inorganic anion transmembrane transporter activity
A0A1D8PK89	37	63,964	General amino-acid permease GAP2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=GAP2 PE=2 SV=1	General amino-acid permease GAP2; amino acid transmembrane transporter activity; amino acid transmembrane transport
G8BH43	28	96,015	Putative uncharacterized protein OS=Candida parapsilosis (strain CDC 317 / ATCC MYA-4646) GN=CPAR2_504150 PE=4 SV=1	A protein complex peripherally associated with the plasma membrane that determines where vesicles dock and fuse. At least eight complex components are conserved between yeast and mammals.
C5MH22	22	53,109	Aureobasidin A resistance protein OS=Candida tropicalis (strain ATCC MYA-3404 / T1) GN=CTRG_05376 PE=4 SV=1	Aureobasidin A resistance protein; inositol phosphoceramide synthase activity; sphingolipid biosynthesis.

* also present in fraction I

processes: carbohydrate metabolism (concentrated filtrate and fraction IV), arginine metabolic pathways (concentrated filtrate and fraction IV), integral cell membrane/wall proteins (all samples), membrane transporters (all samples), DNA metabolism (fraction II), and uncharacterized proteins (all samples) (Table 2, Supplementary Table S2). Notably, none of the proteins identified in the filtrate has recognized antibacterial activity (Table 2, Supplementary Table S2).

When analysing the MS data, the following criteria were assessed for each identified protein: secretion to the growth media, molecular mass (estimated by electrophoretic migration, Supplementary Fig. S3), abundance (estimated from peak area on the chromatogram, Supplementary Fig. S1) and similarity to closest database match (score column in Table 2 and Supplementary Table S2). One protein that appeared to constitute an important secreted yeast product in the examined conditions is glucan exo-1,3-beta-glucosidase (approx. 45 kDa). This enzyme, found in both the concentrated filtrate and the band from the filtrate fraction IV, is involved in carbohydrate metabolism.

Many yeast "killer" toxins active against other yeasts possess glucanase activity that causes increased membrane permeability to ions, which disturbs cell wall stability and disrupts cytoplasmic membrane function [16]. Izgu and Altinbay (2004) [45] showed that the "killer" protein K5 (a glycoprotein) of *Pichia anomala* NCYC 434 is identical to the exo-β-1,3-glucanase of *P. anomala* strain K. This enzyme has a broad killing spectrum among fungi with cell walls predominantly composed of β-1,3-glucan (genera *Candida*, *Torulaspora*, and *Kluyveromyces*). It is stable at pH values of between 3 and 5.5, but loses its activity at higher pH values.

In bacteria, oligosaccharides can form a minor components of the cell envelope that may be secreted from the cell to form mucus or a component of biofilms [46]. Thus glucanases might disturb the structures of both the bacterial cell envelope and biofilms, and this could be responsible for yeast "killer" activity against bacteria. The biofilm formed by the microbial community on the surface of the packing material inside hydrogen-producing bioreactors is an important structure for stability of the process. While our results suggest a significant role for secreted glucanases in disrupting the growth of hydrogen-producing bacteria, other proteins and non-protein compounds produced by ascomycetous yeasts or the product of some synergistic interaction between them may possess antibacterial activity that has yet to be demonstrated. Further studies are necessary to clarify the interactions.

It is necessary to elaborate methods ensuring inhibition of the development of undesirable microorganisms such as killer yeast that inhibit growth of bacteria and performance of hydrogen-yielding bioreactors.

Conclusions

Infection of hydrogen-yielding microbial communities by ascomycetous yeasts has been identified as a major factor inhibiting hydrogen fermentations. Yeast infection of hydrogen-producing bioreactors caused a metabolic shift, a decrease in pH, instability of the microbial community, and a dramatic drop in bio-hydrogen yield. Strains of *Candida humilis*, *Kazachstania exiqua* and *Geotrichum candidum* isolated from the infected bioreactor inhibited the growth of pure cultures of both Gram-positive and Gram-negative bacteria, and the hydrogen-yielding microbial community in acidic environments. Secreted products of the yeast metabolism were shown to inhibit bacterial growth. One candidate as an inhibitory factor is 1,3-beta-glucosidase. Our study clearly shows that elaboration of efficient methods preventing yeast growth in hydrogen-yielding microbial communities are required. It provides another argument that explains why application of dark fermentation as a method for bio-hydrogen production from biomass is still in their infancy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijhydene.2018.05.004.

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