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Co-culturing *Bacillus subtilis* and wastewater microbial community in a bio-electrochemical system enhances denitrification and butyrate formation

Shadi Rahimi^a, Oskar Modin^b, Fariba Roshanzamir^a, Alireza Neissi^a, Soroush Saheb Alam^c, Bastian Seelbinder^d, Santosh Pandit^a, Lei Shi^a, Ivan Mijakovic^{a,e,*}

^a Systems and Synthetic Biology, Chalmers University of Technology, Göteborg, Sweden

^b Division of Water Environment Technology, Department of Architecture and Civil Engineering, Chalmers University of Technology, Gothenburg, Sweden

^c Division of Industrial Biotechnology, Department of Biology and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden

^d Leibniz Institute for Natural Product Research and Infection Biology – Systems Biology and Bioinformatics, Hans Knöll Institute, Adolf-Reichwein-Straße 23, 07745 Jena, Germany

^e The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Lyngby, Denmark

HIGHLIGHTS

- Bio-augmented wastewater with *B. subtilis* rapidly removed nitrate from wastewater.
- Bio-augmentation promoted growth of *Clostridium butyricum* and *C. beijerinckii*.
- Both bio-augmentation and electrical current in BES promoted butyrate production.
- Bio-augmentation combined with BES boost denitrification and butyrate production.

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ABSTRACT

Bio-augmentation could be a promising strategy to improve processes for treatment and resource recovery from wastewater. In this study, the Gram-positive bacterium *Bacillus subtilis* was co-cultured with the microbial communities present in wastewater samples with high concentrations of nitrate or ammonium. Glucose supplementation (1%) was used to boost biomass growth in all wastewater samples. In anaerobic conditions, the indigenous microbial community bio-augmented with *B. subtilis* was able to rapidly remove nitrate from wastewater. In these conditions, *B. subtilis* overexpressed nitrogen assimilatory and respiratory genes including *nasD*, *nasE*, *narG*, *narH*, and *narI*, which arguably accounted for the observed boost in denitrification. Next, we attempted to use the ammonium- and nitrate-enriched wastewater samples bio-augmented with *B. subtilis* in the cathodic compartment of bioelectrochemical systems (BES) operated in anaerobic condition. *B. subtilis* only had low relative abundance in the microbial community, but bio-augmentation promoted the growth of *Clostridium butyricum* and *C. beijerinckii*, which became the dominant species. Both bio-augmentation with *B. subtilis* and electrical current from the cathode in the BES promoted butyrate production during fermentation of glucose. A concentration of 3.4 g/L butyrate was reached with a combination of cathodic current and bio-augmentation in ammonium-enriched wastewater. With nitrate-enriched wastewater, the BES effectively removed nitrate reaching 3.2 mg/L after 48 h. In addition, 3.9 g/L butyrate was produced. We propose that bio-augmentation of wastewater with *B. subtilis* in combination with bioelectrochemical processes could both boost denitrification in nitrate-containing wastewater and enable commercial production of butyrate from carbohydrate-containing wastewater, e.g. dairy industry discharges. These results suggest that *B. subtilis* bio-augmentation in our BES promotes simultaneous wastewater treatment and butyrate production.

1. Introduction

Excessive discharges of various forms of nitrogen into the

environment cause eutrophication of rivers and deterioration of water sources, and by consequence it also increases hazards to human health [1,2]. Therefore, wastewater treatment plants (WWTPs) implement

* Corresponding author at: Systems and Synthetic Biology, Chalmers University of Technology, Göteborg, Sweden.
E-mail address: ivan.mijakovic@chalmers.se (I. Mijakovic).

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various technologies to reduce the concentration of nitrogenous compounds in wastewater before it is discharged into natural bodies of water.

There are several separation-based processes for nitrate removal, including reverse osmosis, ion exchange, electrochemical reduction, electro dialysis, and activated carbon adsorption, which have high operational cost and require disposal of waste brine [3]. The conventional method is to use biological nitrification, i.e. the oxidation of ammonia to nitrite and nitrate, followed by denitrification, which reduces nitrate to nitrogen gas via the following sequence of reactions: $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO}(\text{g}) \rightarrow \text{N}_2\text{O}(\text{g}) \rightarrow \text{N}_2(\text{g})$ [4,5]. Nitrification is carried out by autotrophic microorganisms whereas denitrification is typically carried out by heterotrophic denitrifiers, which use oxidized form of nitrogen as the terminal electron acceptor and organic carbon sources such as glucose, methanol, ethanol, acetic acid, or starch as electron donor [6,7]. There are also emerging technologies for nitrogen removal from wastewater. Anammox bacteria use ammonia as electron donor and nitrite as electron acceptor and produce nitrogen gas and nitrate [8]. Anammox-based technologies are currently applied for treating reject water, which is a side-stream containing high concentrations of ammonia generated during sludge treatment at wastewater treatment plants [9]. Bioelectrochemical system (BES) is another emerging approach for nitrogen removal from wastewater along with energy and/or chemicals production [10]. This technology depends on electrochemically active microorganisms that possess a capacity for extracellular electron transfer to or from a solid electrode (bioanode/biocathode). BES are classified into microbial fuel cells (MFCs) which produce electrical energy and microbial electrolysis cells (MECs), which require an input of electrical energy to drive reactions. Both types have been used for nitrogen removal from wastewater. Gregory et al. (2004) showed that microorganisms could reduce nitrate to nitrite with a cathode as electron donor and Clauwaert et al. (2007) constructed an MFC with a denitrifying biocathode [11,12]. BES have also been used to recover ammonium by charge migration and volatilization in the cathode compartment [13,14].

A variant of BES is called electrofermentation [15]. The electrochemical systems influence the fermentation processes towards production of target chemicals. Cathodic processes can lead to production of more reduced products. For example, Moscoviz et al. (2018) showed that a cathode shifted fermentation products to more 1,3-propanediol during mixed-culture fermentation of glycerol [16]. Pre-colonizing the cathodes with *Geobacter sulfurreducens* appeared to have a positive impact on the overall microbial population shift in the reactor [16].

To improve the performance of biological wastewater treatment and resource recovery processes, bioaugmentation is an interesting approach. Bioaugmentation can involve adding specific microorganisms into a microbial community (MC) to enhance the capacity of the MC for transforming specific contaminants or produce specific products, such as the example with glycerol fermentation mentioned above [16]. Bioaugmentation attempts with pure cultures in wastewater treatment processes have often failed [17]. For example, a nitrifying reactor inoculated with the denitrifying bacterium *Microvirgula aerodenitrificans* failed because the added bacteria were overgrown by protozoa [18]. Ikeda-Ohtsubo et al. (2013) had greater success when using the nitrous-oxide reducing denitrifier *Pseudomonas stutzeri*, which survived for over 32 days and improved denitrification in reactor treating pigery wastewater [19]. It has been pointed out that Bacilli can make an important contribution to transformations of both organic and inorganic nitrogen, due to high nitrate reduction ability and extracellular proteases activity [20]. *Bacillus subtilis* is a well-characterized bacterium amenable for genetic engineering. This makes it especially interesting to study in the context of bioaugmentation. *B. subtilis* could grow in the absence of oxygen using nitrate ammonification and various fermentation processes [21]. In bioelectrochemical systems, *B. subtilis* has been used for oxidation of organic matter and generation of electrical current in anodic compartment [22–24], but there are no

Table 1
Characteristics of wastewaters used in bioelectrochemical systems.

| | Nitrate-enriched wastewater | Ammonium-enriched wastewater |
|---------------------------|-----------------------------|------------------------------|
| pH | 7 | 8.23 |
| NH ₄ -N (mg/L) | 59.60 | 1086 |
| NO ₂ -N (mg/L) | 1.9 | 0.166 |
| NO ₃ -N (mg/L) | 82 | 2.42 |

reports of its role in bioelectrochemical nitrogen removal systems or in electrofermentation.

In this study, we report using *B. subtilis* for bio-augmentation of nitrogen removal and electrofermentation processes. We focus on the ability of *B. subtilis* to grow in reject water and its effect on the indigenous microbial community. Reject water is generated when anaerobically treated sewage sludge is dewatered. It contains high concentrations ammonium, which can account for 25% of the total nitrogen load to a wastewater treatment plant [25]. We cultivated *B. subtilis* in both raw reject water, which we refer to as ammonium-enriched wastewater, and reject water treated with nitrification (ammonia oxidation to nitrite) and anammox [26], which we refer to as nitrate-enriched wastewater (Table 1). The reject water was amended with glucose, which served as carbon source during the experiments. Several experiments were carried out with bioelectrochemical systems to determine if *B. subtilis* could contribute to denitrification in the cathode compartment of such a treatment process and if fermentation of glucose was affected. The results showed that *B. subtilis* bio-augmentation had a positive effect on denitrification and that both bio-augmentation and the cathode in the BES contributed to increased butyrate formation during fermentation of glucose. We argue that this opens up interesting venues for making wastewater treatment more effective energetically and economically.

2. Materials and methods

2.1. Bacterial strains

Bacillus subtilis NCIB3610 comI mutant was applied from Bacillus Genetic Stock Center, with BGSCID 3A38 [27]. *E. coli* DH5 α was used for plasmid construction.

2.2. Genetic manipulation of *B. subtilis*

All PCR primers used in this study are listed in Supplementary Table S1. To examine the effect of assimilatory and respiratory genes, *B. subtilis* overexpressing *nasD*, *nasE*, and *nasF* (OE-NasDEF) was obtained. *Pveg* as the strong constitutive promoter, *nasD*, *nasE*, and *nasF* genes were amplified from *B. subtilis* 168 genomic DNA and then fused as a single fragment with a common *Pveg* promoter for all genes and strong ribosome binding sites (RBS) before each gene. The resulting fragment was inserted into pBS2E (using EcoRI and SpeI restriction sites) [28] and used to transform *E. coli* cells. Transformants were selected on 100 mg/L ampicillin LB plate. The resulting plasmid was used to transform NCIB3610 comI mutant cells, and transformants were selected on 1 mg/L erythromycin LB plate.

To demonstrate the growth of *B. subtilis* in wastewater, *B. subtilis* ypmP-GFP line was used [29].

2.3. Fluorescence microscopy

Cells were examined after 24 h of growth at 37 °C incubator and steady state condition. 1.2% agarose was completely dissolved in Tris-HCl (50 mM) by heating. The microscope slides were floated in agarose solution and then 5 μ l of bacterial suspension was transferred into slides. Then the slides were covered by cover glass and observed

through a LeicaCTR4000 inverted microscope (100× optical magnifications). For examination of the GFP activity, green fluorescence was detected.

2.4. Scanning electron microscopy (SEM)

For SEM imaging, samples were harvested from the liquid–air interface after 24 h of anaerobic culture. Biofilm were fixed with 3% glutaraldehyde for 2 h. The fixed samples were dehydrated with a series of washes with increasing ethanol concentration (40, 50, 60, 70, 80, 90 and 100%) for 10 min each and then dried for 2 h at room temperature. The dried samples were sputter coated with gold (5 nm) before imaging. SEM imaging was performed with the Supra 60 VP microscope (Carl Zeiss AG).

2.5. Aerobic and anaerobic growth condition

A fresh colony grown on an LB plate was used to inoculate 5 ml of LB and grown for overnight at 37 °C. 5×10^6 cells/ml *B. subtilis* was inoculated to 10 ml of nitrate-enriched wastewater (reject water), collected from a municipal wastewater treatment plant in western Sweden. Wastewater without inoculation was used as the control.

To recover the bacterial growth in wastewater, the overnight grown LB culture (5×10^7 cells/ml *B. subtilis*) was used to inoculate 200 µl of wastewater supplemented with different concentrations of glucose (0, 0.5, 1, 2, 4, 8%) in 96 wells plate and incubated with shaking at 250 rpm at 30 °C. Absorbance in 600 nm wavelength was recorded during the culture using EnzyScreen Growth Profiler 960.

2.6. RNA and q-PCR

Samples were harvested and total RNA extraction was performed through RNeasy Mini Kit (Qiagen). For RT-PCR, 1000 ng of total RNA was used as a template for reverse transcription using Quantiscript reverse transcriptase (QuantiTect Reverse Transcription Kit) according to the manufacturer's instructions. To check the gene expression levels, qRT-PCR analyses were conducted in a 20 µl reaction volume with a thermal cycling procedure of denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C/10 s, 60 °C/30 s, and 95 °C/1 min, 55 °C/30 s, 95 °C/30 s. Gene-specific primers were designed to perform the qRT-PCR analysis (Supplementary Table S1). The fluorescent product was detected at the last step of each cycle. The relative quantities of the gene expression levels were assessed using Agilent Technologies Stratagene Mx30005P and were calculated using the comparative cycle threshold (CT) method according to the manufacturers' instructions for normalizing data. A constitutively expressed gene, *16S rRNA* gene was used as an internal reference. Three independent experiments were performed.

Total RNA extraction was performed through RNeasy Mini Kit (Qiagen), and the quality examination was performed by Bioanalyzer (Agilent) through Agilent RNA 6000 Nano Kit. After removing rRNA from total RNA samples through Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina), library was prepared through TruSeq mRNA stranded Bacteria HT (Illumina). RNAseq was performed by paired end reads with 75 bp from each end, and > 100 times coverage per samples, at Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark.

2.7. RNAseq data processing

Pre-processing of paired-end Illumina RNA reads was performed using GEO2RNAseq pipeline [30]. Briefly, quality of reads was analyzed before and after trimming using FastQC (v. 0.11.8). Quality control of reads was performed with Trimmomatic (v. 0.36). Mapping reads to reference genomes was performed using HiSat2. The ammonium and nitrate-enriched sequences were mapped on the reference genome of

the *Clostridium beijerinckii* (NCBI Assembly accession: ASM83310v2; GCF_000833105.2) and *Clostridium butyricum* (NCBI Assembly accession: ASM145606v2; GCF_001456065.2), respectively. In total 284,907,998 2×75 bp sequences were mapped on the reference genomes. Each sample was sequenced four times to ensure sufficient sequencing depth. Samples contained between 2,904,757 and 4,678,797 reads. The SAMtools package was used to convert, sort and index sequence alignment files for downstream data analysis. Gene abundances were estimated per sample using featureCounts tool. The raw data and raw gene counts have been deposited in NCBI's Gene Expression Omnibus [31] and are accessible through GEO Series accession number GSE150480 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150480>). Subsequent RNAseq data processing was performed in R (v. 3.6.1) using a custom-made script, utilizing the Bioconductor package DESeq2 (v. 1.24.0) with the raw gene count (integer) values as input. Counts were normalized using the DESeq2 count function and the normalized parameter changed to TRUE to do estimateSizeFactor function, which scales library sizes between each pair of samples [32]. For each Differential Expression (DE) analysis, low-count genes were removed beforehand; i.e., only genes with at least 10 counts in at least one of the sample groups in design matrix were retained and Log fold change (LFC) calculated for each condition pairs. The gene molecular function collections for *Clostridium beijerinckii* and *C. butyricum* were retrieved from ATGC database [33] using ATGC345 and ATGC350 accession identifiers, respectively. Furthermore, only significantly differentially expressed genes (LFC > 1) were subjected to functional enrichment analysis.

2.8. Nitrate, nitrite and ammonium measurement

Nitrate, nitrite and ammonium were measured using HACH-LANGE® LCK 340, LCK 342 and LCK 304, respectively. Ammonium, nitrite and nitrate removal activities were measured by the Hach spectrophotometer DR 3900 (according to the manufacturer's protocol LCK340 kits for nitrate assay, LCK304 for ammonium assay and LCK342 for nitrite assay).

2.9. Analytical methods

Liquid chromatography systems with UV and RI detectors used for quantification of organic acids and glucose. The HPLC separation was performed on Aminex HPX-87H Bio-Rad ion exclusion column (9 µm, 7.80 mm × 300 mm) with 5 mM H₂SO₄ as the eluent. The flow rate of the mobile phase was 0.6 ml min⁻¹, and pyruvate, succinate, lactate, formate, acetate, and butyrate were monitored at a wavelength of 210 nm and glucose, glycerol, and ethanol were monitored by RI detector.

2.10. MEC design and experimental setup

Two two-chamber H-Cell MECs (Adams & Chittenden Scientific Glass) were used for conducting bioelectrochemical experiments. 50 ml of 10 g/L NaCl and graphite felt anode (3.75 cm² and 2 mm thickness) were used in the anodic chamber. The anode chamber and the cathode chamber (volume 50 ml) were separated using a membrane (Nafion 117, perfluorinated membrane, 0.007in. thick). Wastewater supplemented with 1% glucose, a graphite rod cathode (2.4 cm length and 0.6 cm diameter), an Ag/AgCl (3 M NaCl) reference electrode (Bioanalytical Systems, Inc. BASi) were used as the cathodic compartment. 5×10^6 cells/ml from *B. subtilis* and OE-NasDEF were inoculated to 50 ml of wastewater for *B. subtilis* + MC and OE-NasDEF + MC systems, respectively and MC system was used as the control setup without additional bacteria inoculation. Anode and cathode were contacted via wire (0.025 cm diameter) to the outside of the MEC. The MEC was connected to a two-channel potentiostat/galvanostat (MLab) to record the current and potential during 48 h of run. The MEC was

operated at 37 °C. Nitrogen gas sparged into the cathodic compartment to enhance anaerobic condition.

2.11. 16S rRNA gene sequencing

DNA was extracted from the microbial community formed in cathodic compartments of MECs using DNeasy UltraClean Microbial Kit (Qiagen). The manufacturer's instructions were followed for cell lysis, DNA isolation and purification. The 16S rRNA genes were amplified by PCR with a 341F forward primer and 785R reverse primer pair (Supplementary Table S1). The PCR amplified products were purified using GeneJET PCR Purification Kit (Thermo Scientific). The size and quality of 16S rRNA fragments were checked by agarose gel electrophoresis and nano-drop quality assay. PCR-amplified 16S rRNA samples were shipped in safe-lock Eppendorf tubes and sequenced at Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark.

The pooled sample library underwent paired-end sequencing (2 × 300 bp) on the Illumina MiSeq platform (V3 subunit) to produce up to 30000–40000 paired end reads. The FASTQ files were processed using ezbiocloud bioinformatics service (<https://www.ezbiocloud.net/>) for analysis of the bacterial and archeal community diversity [34]. Processing included merging paired-end reads, trimming primers, filtering by quality, extracting non-redundant reads, taxonomic assignment, detecting chimeras, taxonomic classification and picking Operational Taxonomic Unit (OTU). Following this, species richness was carried out by estimating alpha diversity indices.

Principal coordinate analysis (PCoA) was carried out using genus-level grouping of the OTU table and the Hill-based dissimilarity index of diversity order 1. The calculation was carried out using qdiv [35].

3. Results

3.1. Optimizing growth of *B. subtilis* co-cultured with MC in wastewater with high concentrations of nitrate and ammonium

Bacillus species are known to possess a large gene inventory for nitrate reduction and denitrification, including pathways for dissimilatory nitrate/nitrite reduction to ammonium (DNRA), membrane-bound denitrification, and a periplasmic nitrate reductase [36]. Given this potential for denitrification, we set out to explore the possibility of bio-augmenting the wastewater MC with *B. subtilis* NCIB3610, a well-studied and GRAS model microorganism with robust and sophisticated colony architecture. The choice of this specific strain was made in the hope that its high capacity for biofilm formation (Supplementary Fig. S1a,b) would make it perform well in contact with electrodes in bioelectrochemical systems [37]. We aimed to explore the impact of *B. subtilis* bio-augmentation on denitrification of nitrate-enriched wastewater, containing 40 mg/L nitrate, 16 mg/L nitrite and 111 mg/L ammonium (Fig. 1a). In our experiments, 10 ml samples of wastewater were bio-augmented with 50×10^6 cells of *B. subtilis*. The biomass growth for both the non-augmented (MC) and bio-augmented (*B. subtilis* + MC) samples was slow and limited to about 0.1 OD₆₀₀ (Fig. 1a). It has been previously reported that additional carbon sources can dramatically enhance the growth of heterotrophic MC biomass in wastewater [38]. Therefore, to promote the biomass growth, we supplemented different concentrations of glucose as the organic carbon source to the wastewater (Fig. 1b). Addition of 1% glucose supported optimal biomass growth (Fig. 1b), and hence 1% glucose was supplemented to the wastewater samples in all following experiments. It was already demonstrated that the pre-acclimation strategy using carbon sources significantly affected the microbial community structures [39,40]. Although biomass growth is supported by carbon sources, but it should be likely supplied at optimal concentration to promote growth like 1% glucose in this study. This is consistent with previous report showing number of bacterial strains able to grow decreases with the

increase of the glucose [41].

3.2. Bio-augmentation of MC with *B. subtilis* enhances anaerobic removal of nitrate/nitrite and promotes fermentation

To mimic the conditions typically encountered denitrification processes in WWTPs and benefit from anaerobic nitrate respiration, we shifted our experiments to anaerobic growth conditions (Fig. 2a). Bio-augmentation of the wastewater MC with *B. subtilis* (*B. subtilis* + MC) led to a significantly higher accumulation of biomass at the 6 h time-point, and this effect was no longer observable at 24 h. Concomitant with the biomass accumulation at 6 h, the *B. subtilis* + MC samples also exhibited a peak in nitrate removal (reduction) as well as transient accumulation of nitrite (Fig. 2b, c). Efficient denitrification at the 6 h time point could be attributed to biomass accumulation in *B. subtilis* + MC, whereas both biomass accumulation and denitrification efficiency of *B. subtilis* + MC is close to the control MC system at the 24 h time point. This is consistent with lower nitrite reduction rate compared to nitrate reduction in *B. subtilis*, as previously reported [42,43]. By contrast, there was no significant changes in ammonium concentration in *B. subtilis* + MC during the entire experiment (Fig. 2d).

One explanation for the observed boost of nitrate reduction in *B. subtilis* + MC samples at 6 h could be the expression and activity of *B. subtilis* genes involved in nitrogen metabolism.

Under conditions of nitrogen limitation, *B. subtilis* nitrate and nitrite reductases catalyze the reduction of nitrate via nitrite to ammonia, which becomes a source of nitrogen for cellular anabolism. These enzymes can also support catabolic processes during anaerobic respiration, where nitrate and nitrite become terminal electron acceptors. The respiratory *B. subtilis* nitrate reductase is encoded by *narGHI* [44], and the assimilatory nitrate reductase is encoded by *nasBC* [45]. The *nasBC* operon is co regulated with a nitrate transporter gene, *nasA* [46]. Unlike two distinct nitrate reductases, *B. subtilis* possesses only one NADH-dependent nitrite reductase, encoded by *nasDE* [47]. This nitrite reductase is involved in both the assimilatory and the respiration processes and requires NasF for cofactor formation [47]. When *B. subtilis* was co-cultured with wastewater MC in aerobic conditions (*B. subtilis* + MC), we observed a slight but significant overexpression (2–5-fold) of *B. subtilis* *nasB*, *nasC*, and *nasD* compared to *B. subtilis* growing in the LB medium (Fig. 2e). In anaerobic conditions, the expression of *B. subtilis* respiratory genes *nasD*, *nasE*, *narG*, *narH* and *narI* was strongly enhanced at the 6-h time-point (2.5–120-fold) (Fig. 2f). This is consistent with previous findings claiming that nitrite reductase activity in *B. subtilis* gets strongly induced under oxygen limitation [48]. Anaerobic induction of *nasDEF* expression via the ResDE two-component system requires the presence of nitrite, which is in accord with the transient nitrite accumulation observed in our study (Fig. 2c). We therefore concluded that the peak of nitrate reduction in the *B. subtilis* + MC samples (observed at 6 h), and subsequent nitrite reduction (up to 24 h), correlate to overexpression of *B. subtilis* nitrate/nitrite respiratory genes.

Next, we assessed the fermentation capacity of biomass in the MC and *B. subtilis* + MC samples under anaerobic conditions (Fig. 2g–m). Glucose consumption was comparable in both samples. At the 6-h time-point, the *B. subtilis* + MC samples had a significant advantage in terms of acetate (Fig. 2h), lactate (Fig. 2i) and pyruvate (Fig. 2k) production, while the difference in ethanol production was not significant. At 24 h, the *B. subtilis* + MC samples kept the advantage in terms of lactate production and produced more succinate (Fig. 2l). However, they lost the advantage in terms of acetate and pyruvate production and produced significantly less butyrate (Fig. 2j). Overall, these results indicate that, in addition to stimulating nitrate/nitrite reduction, bio-augmentation of MC with *B. subtilis* also promotes generation of some valuable fermentation products. These two effects are likely to be related, since it was previously found that the distribution of end-products in anaerobic fermentation is affected by redox processes and specifically the

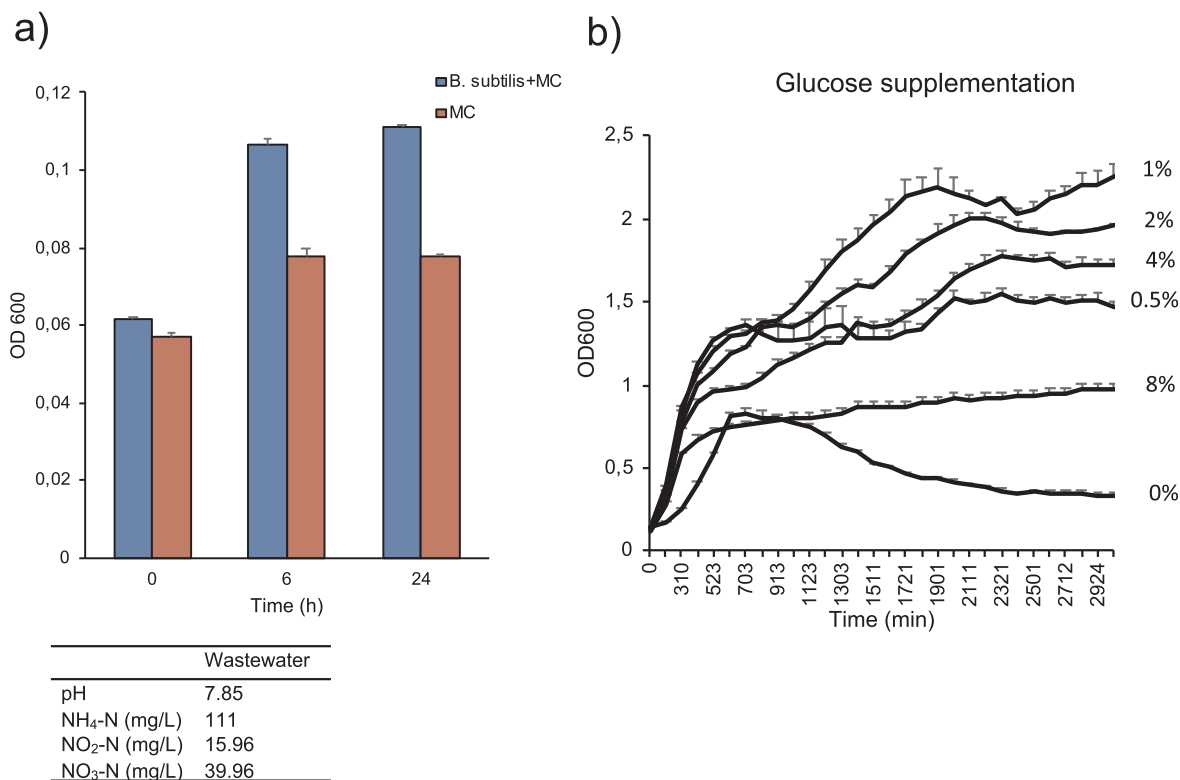


Fig. 1. Growth of cocultured *B. subtilis* with wastewater microbial community at high concentrations of nitrate and ammonium present in wastewater with supplementation of glucose. a. *B. subtilis* added to the wastewater including microbial community (*B. subtilis* + MC) and the biomass growth was measured by optical density at 600 nm wavelength and compared with the biomass of wastewater microbial community (MC). Wastewater characteristics are mentioned in a table below the graph. b. *B. subtilis* cocultured with microorganisms present in wastewater supplemented with different percentages of glucose (0, 0.5, 1, 2, 4, 8%). The biomass concentration was measured by the optical density at 600 nm wavelength.

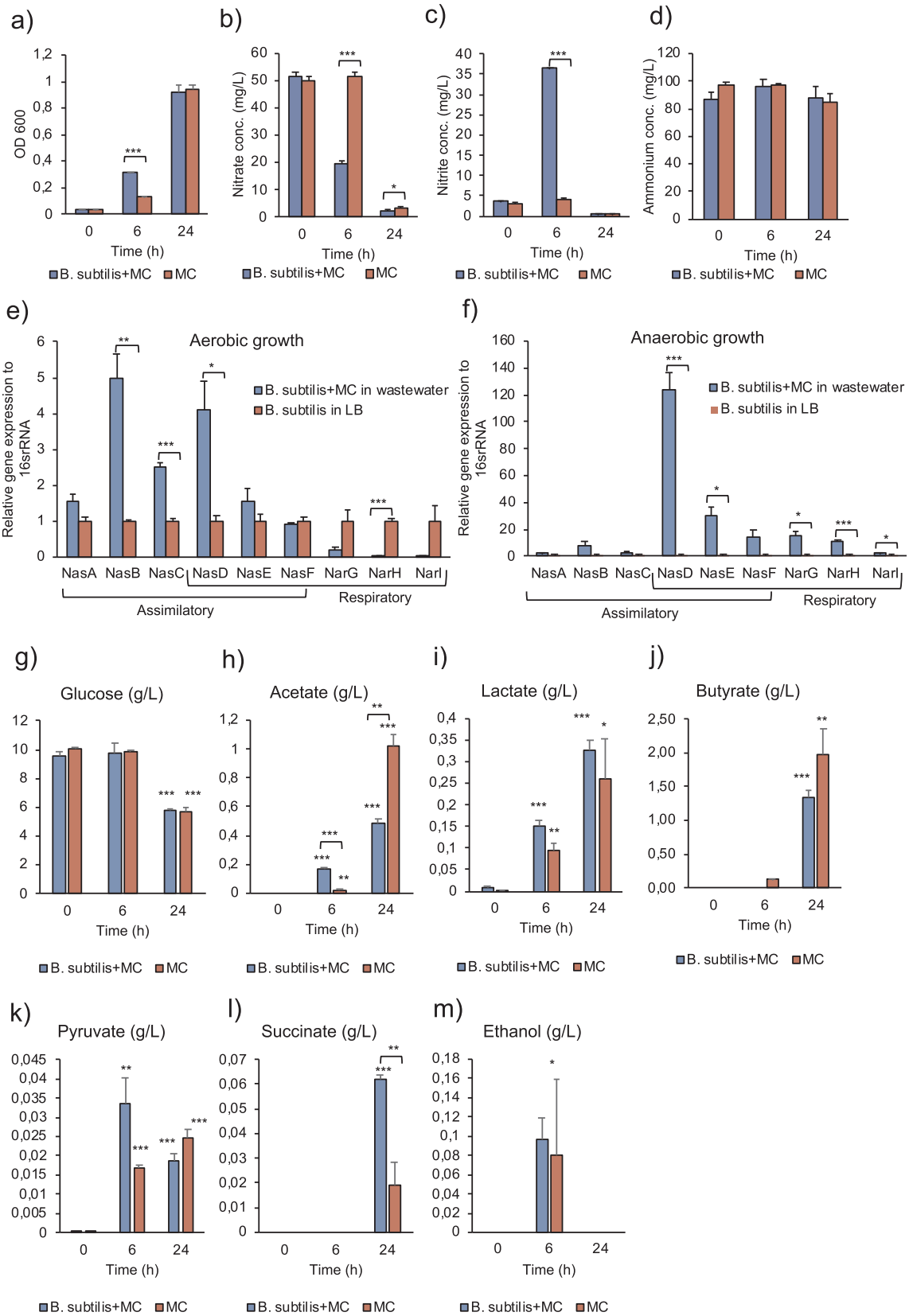
biological redox couple NAD^+/NADH (Saint-Amans et al., 2001).

3.3. Bio-augmentation of wastewater MC with *B. subtilis* in cathodic MECs has minimal effect on nitrogen removal

To examine any possible effects of bio-augmentation with *B. subtilis* in bioelectrochemical systems for wastewater treatment, we carried out experiments with both nitrate- and ammonium-enriched wastewaters in the cathodic compartment (Fig. 3). Experiments were conducted both with the cathode potential controlled at -1 V vs a Ag/AgCl reference electrode, which refer to as connected, and with open-circuit conditions, which we refer to as disconnected. In the connected experiments, electrons delivered from the cathode could contribute to denitrification in the system. To further clarify the contribution of *B. subtilis*, we focused on the *nasDEF* operon encoding NADH-dependent nitrite reductase as the most highly induced gene during anaerobic nitrate respiration (Fig. 2f) [49]. To observe the effect of *B. subtilis* nitrogen metabolism on nitrogen removal and fermentation, we prepared a genetically engineered *B. subtilis* in which the genes for the NADH-dependent nitrite reductase (*nasDEF*) were overexpressed. This strain was also used for bio-augmentation of the MC, in samples labelled as OE-NasDEF + MC. When we used ammonium- and nitrate-enriched wastewaters at the cathode, in both cases cathodic reactions resulted in a pH drop starting from the 18 h time point which may be due to fermentation at the cathode (Supplementary Fig. S2a,b). Cathodic current production increased at 18 h in MECs with both ammonium- and nitrate-enriched wastewaters (Supplementary Fig. S2c, d). Lower pH would facilitate the reduction of H^+ to H_2 on the cathode surface. However, ammonium-enriched wastewater produced higher cathodic current, especially with *B. subtilis* + MC, despite having a higher pH

than nitrate-enriched wastewater. This suggests that the bacterial community mediated electron transfer from the cathode. This electron transfer may lead to more NADH availability for cellular metabolism in the *B. subtilis* + MC system for fermentation.

Regarding nitrogenous compounds in ammonium-enriched wastewater, minimal differences were observed in removal of nitrate, nitrite, or ammonium concentrations between non-augmented and bio-augmented samples (Supplementary Fig. S2e–g). In case of nitrate-enriched wastewater, nitrate was reduced in all MECs, however, the reduction was somewhat slower in the OE-NasDEF + MC connected system (Supplementary Fig. S2h). Nitrite concentration was also reduced in all MECs after 48 h, with a transient nitrite accumulation observed especially in OE-NasDEF + MC samples at the 6 h time point (Supplementary Fig. S2i). In case of ammonium removal, a difference was observed with the *B. subtilis* + MC connected samples, which removed ammonium faster than other samples, reaching 93% removal after 48 h which may be due to ammonium assimilation (Supplementary Fig. S2j). Diffusion through the ion exchange membrane into the anode compartment contributed to ammonium removal in all experiments, meanwhile some part of ammonium from ammonium-enriched wastewater diffused to the anode are transported to the cathode due to the driving force of electric field for migration of positively charged ions such as ammonium [50] (Supplementary Fig. S3). However, the connected OE-NasDEF + MC had only negligible ammonium concentrations in the anode compartment, suggesting ammonium assimilation and possibly nitrification/denitrification (slow nitrate reduction and transient nitrite accumulation) were responsible for ammonium removal (Supplementary Fig. S3d). A possible explanation for increased assimilation could be that the electric connection positively influenced the OE-NasDEF strain to assimilate more ammonium



(caption on next page)

Fig. 2. Efficient growth of *B. subtilis* cocultured with wastewater microbial community and nitrate removal from wastewater in anaerobic condition along with upregulation of assimilatory and respiratory genes and changes in fermentation products. a Anaerobic biomass growth of *B. subtilis* cocultured with wastewater microbial community (*B. subtilis* + MC) supplemented with 1% glucose was compared with that of microbial community (MC). The biomass concentration was measured by the optical density at 600 nm wavelength. b–d Nitrate, nitrite, and ammonium removal activity in *B. subtilis* + MC were compared with microbial community of wastewater MC. e, f Relative expression level of *B. subtilis* assimilatory and respiratory genes (*nasA*, *nasB*, *nasC*, *nasD*, *nasE*, *nasF*, *narG*, *narH*, *narI*) was measured by quantitative reverse-transcriptional PCR in *B. subtilis* cocultured system in wastewater and LB medium in aerobic and anaerobic growth condition. 16S rRNA gene was used as the reference gene. g–m glucose and fermentation products were analyzed in anaerobic *B. subtilis* + MC and MC in wastewater. Data represent the mean \pm SE of three independent replicates and it was statistically analyzed and compared with the control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) using Student's *t* test.

through supplying more ATP for the glutamine biosynthesis via the following reaction catalyzed by glutamine synthetase; Glutamate + ATP + NH₃ → Glutamine + ADP + phosphate.

3.4. Bio-augmentation of wastewater MC with *B. subtilis* in cathodic MECs promotes fermentation processes, leading to accumulation of products such as butyrate, acetate and ethanol

Next, we examined the accumulation of products of cathodic-space fermentation, including pyruvate, succinate, lactate, glycerol, formate, acetate, ethanol, and butyrate (Fig. 4, Supplementary Fig. S4). We compared disconnected and connected samples of MC, *B. subtilis* + MC and OE-NasDEF + MC, growing in ammonium- and nitrate-enriched wastewater.

Overall, the MC could produce more fermentation products in ammonium-enriched wastewater compared with nitrate-enriched wastewater (Fig. 4, Supplementary Fig. S4). In ammonium-enriched wastewater, butyrate formation was stimulated by bio-augmentation of MC with *B. subtilis*, and to a lesser extent with *B. subtilis* OE-NasDEF (Fig. 4a, Supplementary Fig. 4b). For all other fermentation products, bio-augmentation either had no effect (acetate, Supplementary Fig. 4c) or attenuated their accumulation (pyruvate, Supplementary Fig. 4e; succinate, Supplementary Fig. 4f; ethanol, Supplementary Fig. 4d). Interestingly, a decrease in accumulation of some specific fermentation products was related only to bio-augmentation with *B. subtilis* OE-NasDEF: such was the case of formate (Supplementary Fig. 4h) and to a lesser extent lactate (Supplementary Fig. 4g).

In nitrate-enriched wastewater, bio-augmentation with wild type *B.*

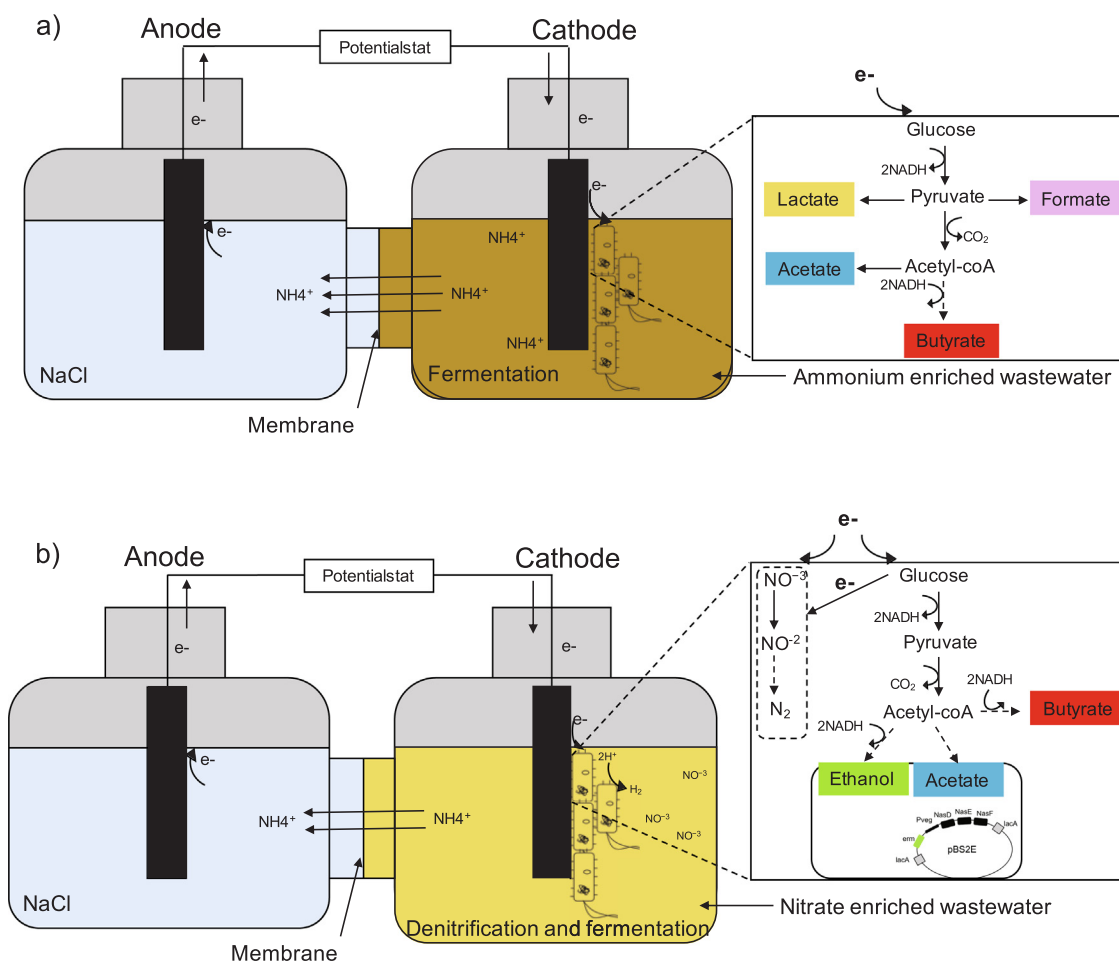


Fig. 3. The schematic summary of nitrogen removal and fermentation using ammonium- and nitrate-enriched wastewaters. a Cathodic ammonium removal, anodic ammonium recovery, butyrate formation as well as formate, acetate and lactate by-production by connected *B. subtilis* cocultured MEC using ammonium-enriched wastewater. b Cathodic nitrate and ammonium removal, anodic ammonium recovery, butyrate formation as well as hydrogen generation by connected *B. subtilis* cocultured MEC using nitrate-enriched wastewater. Acetate and ethanol production in OE-NasDEF cocultured system in connected and disconnected systems using nitrate-enriched wastewater.

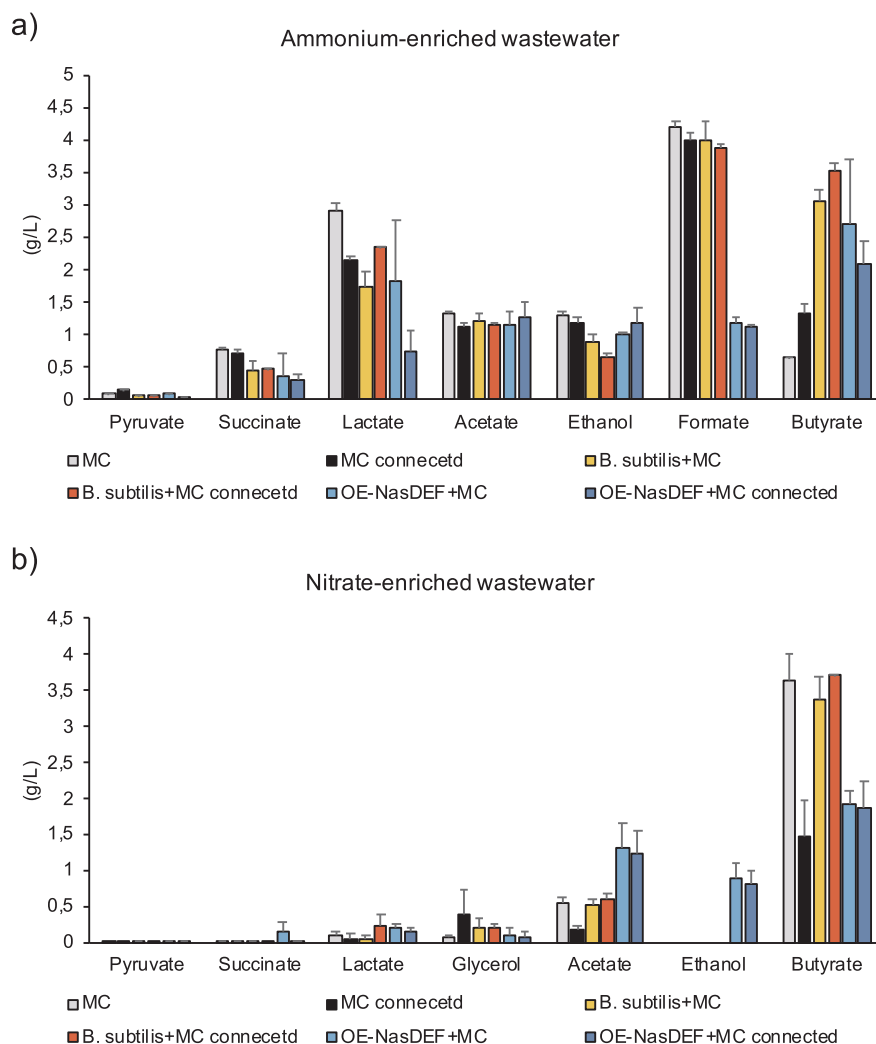


Fig. 4. Butyrate formation in cathodic MEC of *B. subtilis* cocultured (*B. subtilis* + MC) system using ammonium- and nitrate-enriched wastewater after 42 h time point. Glucose and fermentation products were analyzed using High-performance liquid chromatography (HPLC) in samples collected from cathodic compartment of disconnected and connected MECs of *B. subtilis* cocultured (*B. subtilis* + MC), microbial community (MC), and OE-NasDEF cocultured (OE-NasDEF + MC) using ammonium and nitrate-enriched wastewaters. a) Butyrate, formate, acetate, pyruvate, succinate, lactate, ethanol were measured after 42 h from disconnected and connected cathodic MECs of *B. subtilis* + MC, MC, and OE-NasDEF + MC using ammonium-enriched wastewater. b) Acetate, ethanol, butyrate, pyruvate, succinate, lactate, glycerol were measured after 42 h from disconnected and connected cathodic MECs of *B. subtilis* + MC, MC, and OE-NasDEF + MC using nitrate-enriched wastewaters. Data represent the mean \pm SE of three independent replicates.

subtilis did not have a pronounced effect on accumulation of fermentation products (Fig. 4b), but the presence of *B. subtilis* OE-NasDEF strongly stimulated the accumulation of acetate (Supplementary Fig. 4k, Supplementary Fig. S5a), ethanol (Supplementary Fig. 4l, Supplementary Fig. S5d) and to some extent succinate (Supplementary Fig. 4n). Glycerol (Supplementary Fig. 4p) and butyrate (Supplementary Fig. 4j) accumulation was attenuated in the presence of *B. subtilis* OE-NasDEF. In terms of current production, no notable differences were observed among the tested samples (Supplementary Fig. S2d).

In most cases, connecting the MECs typically did not have a profound effect on accumulation of fermentation products. However, the presence of bacteria had an impact on hydrogen production by the biocathode in the nitrate-enriched wastewater. Cyclic voltammetry (CV) showed that 48 h cultivation lead to high hydrogen production at more positive potential suggesting that the presence of microorganisms catalyzed hydrogen generation on the electrode (Supplementary Fig. S6j–l). With ammonium-enriched wastewater, only the OE-NasDEF + MC connected samples showed better hydrogen generation catalysis after 48 h (Supplementary Fig. S6f). Therefore, along with butyrate formation, formate, acetate, ethanol, and lactate were the major by-products of fermentation using ammonium-enriched wastewater, whereas H_2 production can be considered as the main by-product of fermentation using nitrate-enriched wastewater (Fig. 3).

Bio-augmentation with *B. subtilis* obviously had a stimulating

impact on accumulation of fermentation products. However, the wild type *B. subtilis* and the OE-NasDEF strains exhibited some remarkable differences in terms of metabolite profiles. Next, we set out to explore these differences.

3.5. Bio-augmentation with *B. subtilis* in cathodic MECs triggers profound changes in species composition of wastewater MCs

In order to assess whether the contribution of *B. subtilis* bio-augmentation to the MC fermentation measured in our experimental MEC setup (Fig. 4, Supplementary Fig. 4) is direct (direct metabolic activity of *B. subtilis*) or may also be indirect (changes in MC composition), we determined the composition of the MC by 16S rRNA gene sequencing (Fig. 5). The sequencing was performed on samples after 18 h in the cathodic space of MECs, since this was the period of most pronounced changes in terms of electricity generation and fermentation profiles.

Principal coordinate analysis (PCoA) showed that the type of wastewater had a strongest effect on the microbial community (Supplementary Fig. S7). However, bioaugmentation with *B. subtilis* also had a clear effect and was responsible for the separation along the second principal coordinate (PC2). In case of ammonium-enriched wastewater, connecting the MEC system even in the absence of *B. subtilis* had a pronounced effect on species composition (Fig. 5a). Dominant MC genera such as *Streptococcus* (with *Streptococcus parasuis* as the most abundant species, comprising 2% of the total MC) and

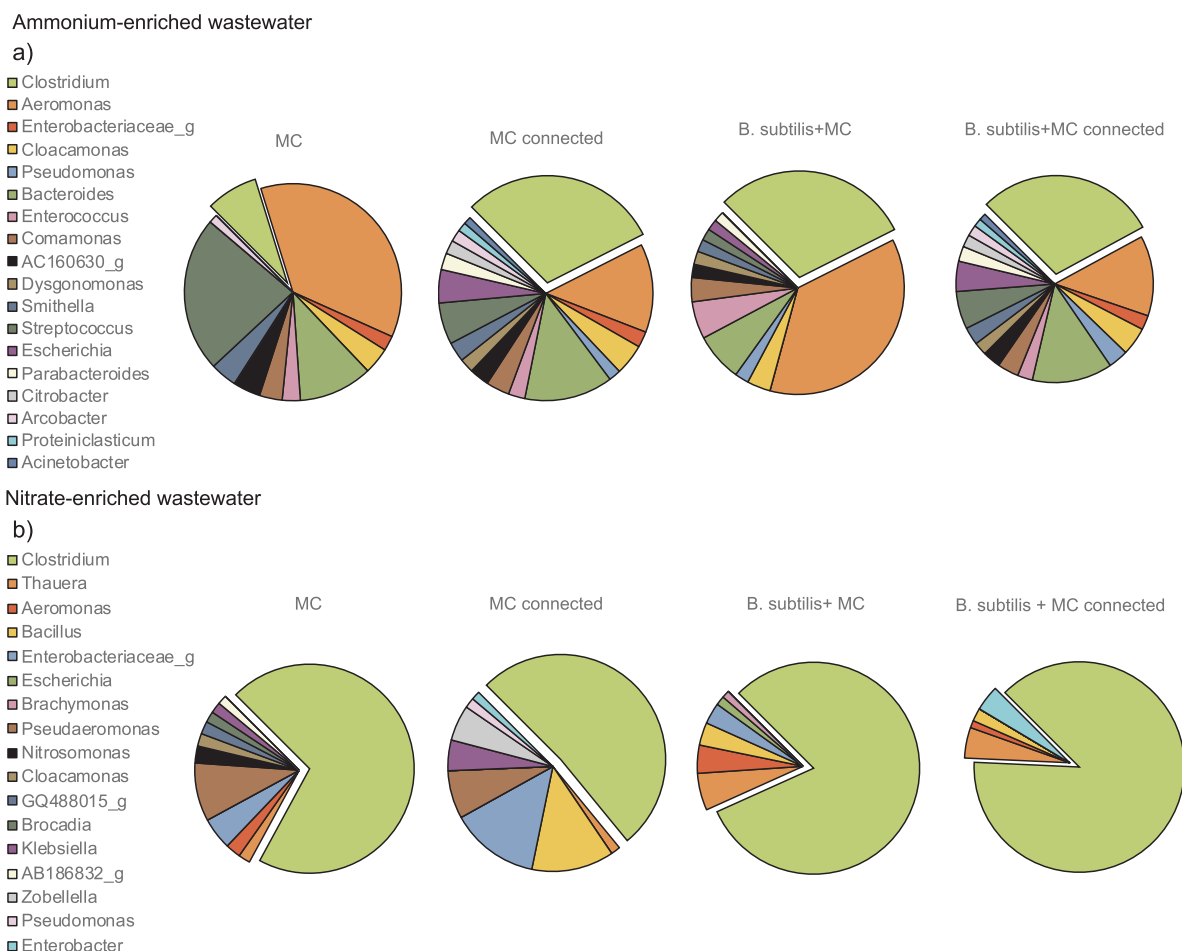


Fig. 5. Interactive stimulatory effect of *B. subtilis* coculture on growth of *Clostridium* using ammonium and nitrate-enriched wastewaters in cathodic MECs after 18 h. 16S rRNA gene fragment was amplified and sequenced using genomic DNA extracted from disconnected and connected *B. subtilis* cocultured (*B. subtilis* + MC) and microbial community (MC) samples in ammonium and nitrate-enriched wastewaters after 18 h. a, b Bacterial population (phylum and genus) in *B. subtilis* + MC and MC disconnected and connected systems using ammonium-enriched wastewater. c, d Bacterial population (phylum and genus) in *B. subtilis* + MC and MC disconnected and connected systems using nitrate-enriched wastewater.

Aeromonas were strongly attenuated upon establishing the electrical connection. By contrast, the connection promoted a slight increase of *Bacteroides* (12% of the total MC) and a dramatic increase of *Clostridium* species (26% of the total MC, with *C. butyricum* as the most abundant species comprising 14% of the total MC). Interestingly, bio-augmentation of the non-connected MC with *B. subtilis* provoked similar effects as connecting the non-augmented system: promotion of growth of *Clostridium* species (27% of the total MC, with *C. butyricum* as the most abundant species with 17.5% of the MC) and attenuation of *Streptococcus* (*S. parasuis* being the most abundant species with 1% of the MC). However, *Aeromonas* was not affected by bio-augmentation with *B. subtilis* without an electrical connection. The connected bio-augmented MC had a genera composition very similar to the connected non-augmented MC (26% *Clostridium*, 11.7% *Aeromonas*, 12% *Bacteroides*). The most abundant species in the connected bio-augmented MC were *C. butyricum* (17.5% of the total MC), *Bacteroides graminisolvens* (12% of the total MC) and *Aeromonas simiae* (6% of the total MC).

In nitrate-enriched wastewater the starting MC community had a very different composition, with *Clostridium* accounting for 54% of the MC with *C. beijerinckii* being the most abundant species (26% of the total MC) (Fig. 5b). Connecting the system reduced the *Clostridium* genus to 46% (*C. beijerinckii* to 14% of the total MC), but the genus still remained dominant. Enterobacteriaceae (group G) and *Bacillus* increased their contribution to the MC to 12% and 11%, respectively, with *B. funiculus* being the only identified species of this genus. Bio-

augmentation of the non-connected MC with *B. subtilis* mainly increased the *Clostridium* fraction to 70% of the total MC, and the most abundant species became *C. butyricum* with 22% of the total MC. Species composition of the *B. subtilis* + MC sample was not significantly altered upon connection.

B. subtilis 16S rDNA was not found in non-augmented MC in ammonium- and nitrate-enriched wastewaters. It was only detected in bio-augmented MC (2.9% of the total MC) in nitrate-enriched wastewater but not in the ammonium-enriched wastewater (Supplementary Table S3, Supplementary Fig. S8e, f). Upon electrical connection, *B. subtilis* 16S rDNA was not detected in either ammonium- or nitrate-enriched wastewaters. This suggests that bio-augmentation of *B. subtilis* in most cases may play an indirect role, by triggering and promoting growth of other species via metabolic cross-feeding in the early phase of the coculture. Species that especially profited from the presence of *B. subtilis* were *C. butyricum* and *C. beijerinckii*, which became dominant in ammonium- and nitrate-enriched wastewaters, respectively.

3.6. Bio-augmentation of wastewater MC with *B. subtilis* affects expression of *Clostridia* genes involved in cathodic reactions, corresponding to the observed changes in the fermentation profiles

Our 16S rDNA analysis pointed out to *C. butyricum* and *C. beijerinckii* as the dominant species in bio-augmented MCs, using ammonium- and nitrate-enriched wastewaters, respectively. Therefore, we hypothesized

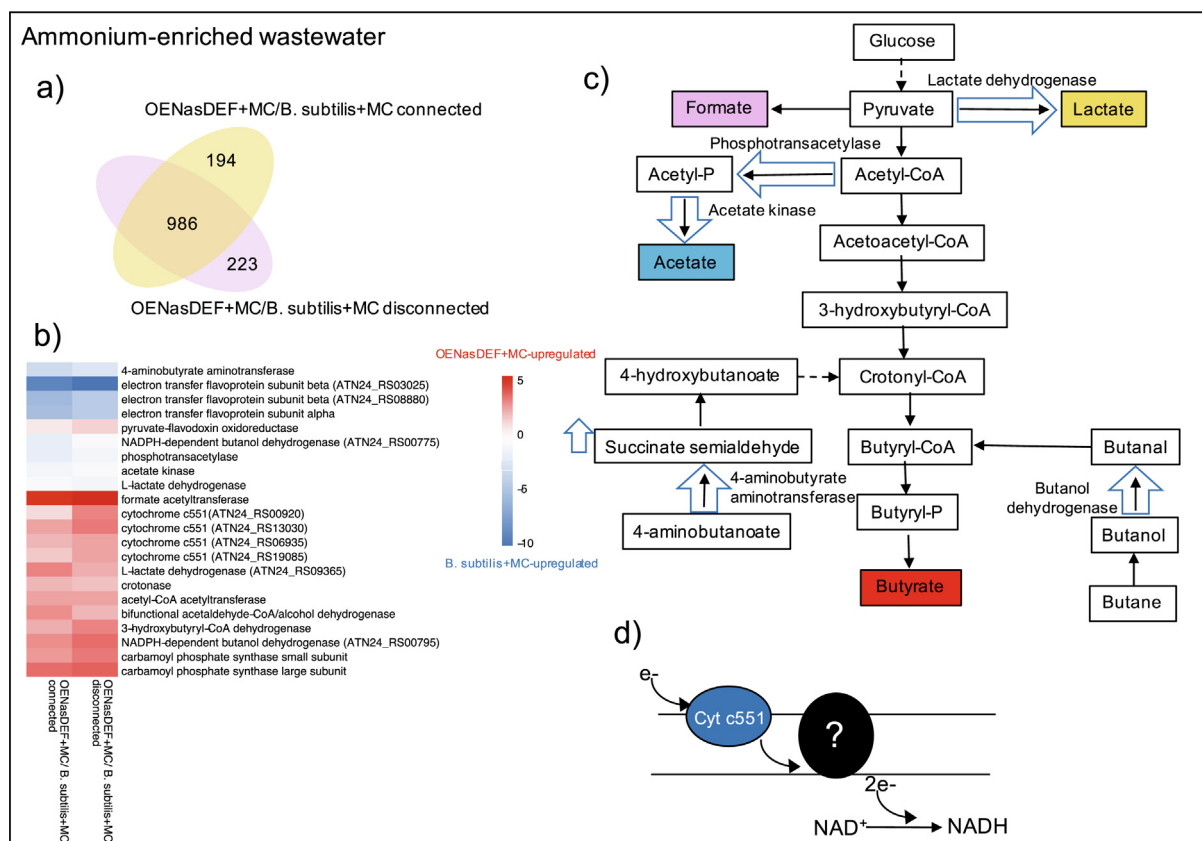


Fig. 6. The differentially expressed genes of *C. butyricum* in cathodic MEC using ammonium-enriched wastewater. a Venn diagram of *C. butyricum* differentially expressed genes (log fold change (LFC) > 1 among two different conditions, OE-NasDEF + MC/*B. subtilis* + MC disconnected: disconnected OE-NasDEF + MC compared to disconnected *B. subtilis* + MC, OE-NasDEF + MC/*B. subtilis* + MC connected: connected OE-NasDEF + MC compared to connected *B. subtilis* + MC) in ammonium-enriched wastewater, b *C. butyricum* differentially expressed genes of selected gene subset in Supplementary Table S4 (LFC > 1 among two different conditions in a) in ammonium-enriched wastewater, c schematic diagram of upregulated *C. butyricum* genes influencing fermentation in ammonium-enriched wastewater, d schematic diagram of *C. butyricum* upregulated electron transferring genes in ammonium-enriched wastewater.

that these dominant species may have a considerable impact on the observed accumulation of fermentation products (Fig. 4, Supplementary Fig. 4). To assess this, we quantified the expression of the genes of these two species involved in cathodic reactions potentially related to accumulation of fermentation products (Figs. 6 and 7). RNAseq analysis was performed on samples harvested from disconnected and connected *B. subtilis* + MC and OE-NasDEF + MC in ammonium- and nitrate-enriched wastewaters, at the 18 h time-point. *C. butyricum* RNAseq analysis was performed in ammonium-enriched wastewater, and *C. beijerinckii* RNAseq analysis in nitrate enriched wastewater. The data were analyzed with the objective to clarify the observed differences between the bio-augmentation with the wild type *B. subtilis* and *B. subtilis* OE-NasDEF when it comes to influencing the fermentation profile of the MC (Fig. 4, Supplementary Fig. 4). For this purpose, we determined the *C. butyricum* and *C. beijerinckii* differentially expressed genes (DEGs) (log fold change (LFC) > 1) in OE-NasDEF + MC compared to *B. subtilis* + MC in disconnected (OE-NasDEF/*B. subtilis* disconnected) and connected conditions (OE-NasDEF/*B. subtilis* connected).

In ammonium-enriched wastewater, we found a large number of DEGs (986) to be present in both *C. butyricum* DEGs datasets (OE-NasDEF + MC/*B. subtilis* + MC disconnected and OE-NasDEF + MC/*B. subtilis* + MC connected) (Fig. 6a). Since we observed increased levels of butyrate in *B. subtilis* + MC using ammonium-enriched wastewater, we focused on a subset of genes of *C. butyricum* potentially related to butyrate formation, electron transfer and carbamoyl phosphate synthase (involved in ammonium metabolism) (Supplementary Table S4). Among these genes, upregulation of *C.*

butyricum 4-aminobutyrate aminotransferase and butanol dehydrogenase genes (both contribute in producing butyrate precursors) was detected in *B. subtilis* + MC, suggesting their contribution to excessive butyrate formation in ammonium-enriched wastewater (Fig. 6b, c). Upregulated *C. butyricum* phosphotransacetylase, acetate kinase and lactate dehydrogenase could be correlated with acetate and lactate accumulation detected in *B. subtilis* + MC samples (Fig. 6b, c). Furthermore, higher expression of *C. butyricum* cytochrome c551 isoforms and electron transfer flavoprotein beta subunit suggested increased electron transfer from the electrode to the MC in both *B. subtilis* + MC and OE-NasDEF + MC samples (Fig. 6b, d).

In nitrate-enriched wastewater, only 135 common DEGs were found in two *C. beijerinckii* DEGs datasets (OE-NasDEF + MC/*B. subtilis* + MC disconnected and OE-NasDEF + MC/*B. subtilis* + MC connected). This indicated that DEGs varied much more between connected/disconnected conditions in nitrate enriched wastewater (Fig. 7a). As a consequence of the electrical current, significant cathodic ammonium removal and less anodic ammonium recovery was observed specifically in the connected OE-NasDEF + MC samples, suggesting ammonium assimilation by the MC of the nitrate-enriched wastewater. In the connected OE-NasDEF + MC system we detected significant over-expression of *C. beijerinckii* genes encoding nitrogenase, nitrogenase iron protein, nitrogen fixation protein and a glutamine ABC transporter (Supplementary Fig. S10). By comparison, expression of these genes was much lower in the connected *B. subtilis* + MC system (Supplementary Fig. S10). Furthermore, in the connected OE-NasDEF + MC we observed up-regulation of the *C. beijerinckii* glutamine synthetase (catalyzed glutamine biosynthesis from ammonia and

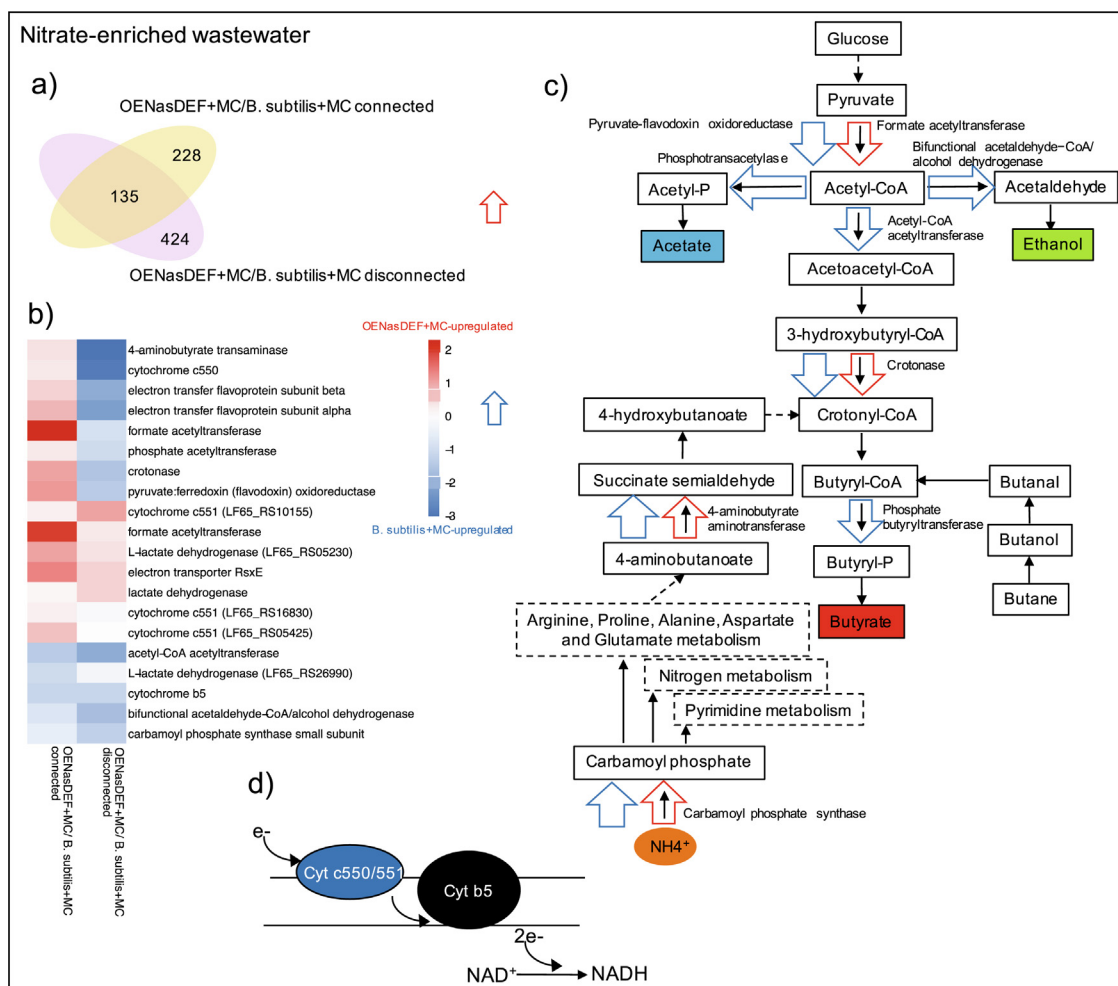


Fig. 7. The differentially expressed genes of *C. beijerinckii* in cathodic MEC using nitrate-enriched wastewater. a Venn diagram of *C. beijerinckii* differentially expressed genes (LFC > 1 among two different conditions, OE-NasDEF + MC/*B. subtilis* + MC disconnected: disconnected OE-NasDEF + MC compared to disconnected *B. subtilis* + MC, OE-NasDEF + MC/*B. subtilis* + MC connected: connected OE-NasDEF + MC compared to connected *B. subtilis* + MC) in nitrate-enriched wastewater, b *C. beijerinckii* differentially expressed genes of selected gene subset in Supplementary Table S5 (LFC > 1 among two different conditions in a) in nitrate-enriched wastewater, c schematic diagram of upregulated *C. beijerinckii* genes influencing fermentation in nitrate-enriched wastewater, d schematic diagram of upregulated *C. beijerinckii* electron transferring genes in nitrate-enriched wastewater.

glutamate), 4-aminobutyrate aminotransferase (involved in amino acid metabolism) and carbamoyl phosphate synthase (involved in nitrogen disposal through the urea cycle and pyrimidines synthesis). This is consistent with active assimilation of ammonium by *C. beijerinckii*, explaining the diminished anodic ammonium recovery in the OE-NasDEF + MC connected system (Fig. 7b, c, Supplementary Fig. S3d). Since we observed butyrate accumulation in *B. subtilis* + MC samples and acetate and ethanol accumulation in OE-NasDEF + MC samples using nitrate-enriched wastewater, we examined the *C. beijerinckii* gene subset involved in butyrate, acetate, and ethanol formation, electron transfer and carbamoyl phosphate synthase genes (involved in ammonium metabolism) (Supplementary Table S5). In *B. subtilis* + MC, we observed overexpression of *C. beijerinckii* genes encoding crotonase, pyruvate:ferredoxin (flavodoxin) oxidoreductase, phosphate butyryltransferase, acetyl-CoA acetyltransferase, 4-aminobutyrate transaminase, and formate acetyltransferase: all contributing to butyrate formation (Fig. 7b, c). However, we also detected overexpression of *C. beijerinckii* genes encoding phosphate acetyltransferase, acetate kinase, bifunctional acetaldehyde-CoA/alcohol dehydrogenase genes in *B. subtilis* + MC, all of which are involved in acetate and ethanol accumulation (Fig. 7b, c). Since no extra accumulation of acetate and ethanol was detected in *B. subtilis* + MC samples, it is possible that the acetate and ethanol were increasingly consumed by metabolic cross-

feeding in the complex MC. Interestingly, we did not find any evidence of overexpression of genes for acetate and ethanol biosynthesis in OE-NasDEF + MC samples. Finally, cytochromes b5, c550, c551 and the electron transfer flavoprotein alpha and beta subunits were all overexpressed in connected *B. subtilis* + MC and OE-NasDEF + MC samples, suggesting that enhanced electron transfer from the electrode could have affected the *C. beijerinckii* metabolism (Fig. 7b, d).

4. Discussion

In this study we established that *B. subtilis* can be used for bio-augmentation of the MC in reject water; however, addition of glucose was needed to promote growth. Bio-augmentation stimulated nitrate/nitrite reduction and generation of some valuable fermentation products, such as butyrate. Although *B. subtilis* did not have high relative abundance in the MC, it affected the MC composition and metabolism.

With nitrate-enriched wastewater in the cathodic compartment, *B. subtilis* bio-augmentation stimulated denitrification (96% nitrate removal, reaching 3.2 mg/L nitrate). It is consistent with the beneficiary effect of *B. subtilis* on nitrate reduction with upregulation of nitrogen assimilatory and respiratory genes (Fig. 2b, f).

Another interesting observation was that bio-augmentation of the MC with *B. subtilis* led to a significant reduction of pyruvate levels and

improved formation of butyrate during glucose fermentation (Supplementary Fig. 4b, Supplementary Fig. S9a). As suggested by previous reports [51], increased butyrate production in bioelectrochemical systems can be related to increased electron supply through the cathode, providing extra reducing power and higher NADH levels. Our results suggest that the observed effect of *B. subtilis* bio-augmentation on butyrate production was mainly indirect. To a large extent it depended on promoting growth of *C. butyricum* and *C. beijerinckii* which became the dominant species in the community (Supplementary Table S2). *Clostridium* genus, including *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii*, *C. populeti*, and *C. thermobutyricum*, is the most commonly used source for production of butyric acid, with acetate, CO₂ and H₂ as the main by-products [52–56]. We are not the first to report this type of effect in co-culture. There are several studies on syntrophic cocultures of *Bacillus* and *Clostridium* species for the purpose of producing acetone, ethanol, butanol, butyric acid, biofuels and biohydrogen [57–65]. Compatibility of *B. subtilis* with strictly anaerobic *Clostridium* sp., such as *C. beijerinckii*, was also previously reported [66]. In a microbial co-culturing system, *Bacillus* species grow with oxygen consumption and this favors anaerobic growth of *Clostridium* species. Furthermore, *Clostridium* species have been shown to utilize the hydrolyzed glucose provided by *B. subtilis* growing on starchy-based feedstocks and use this as a carbon source for production of acetone–butanol–ethanol [66]. However, this cross-feeding between *Bacillus* and *Clostridium* species still required supplementation of mixed nitrogen sources, such as the yeast extract and NH₄NO₃ [62,63]. In our current study, the nitrogen source was provided by the cost-free wastewater samples.

Our RNAseq analysis correlated butyrate accumulation to overexpression of corresponding metabolic pathways in *C. butyricum* and *C. beijerinckii*, the dominant species in *B. subtilis* + MC samples. By contrast, the RNAseq analysis could not explain the observed effects on acetate and ethanol production in OE-NasDEF + MC samples. The simplest explanation for this is that other bacteria present in the MC may have been affected by bio-augmentation with OE-NasDEF and contributed to acetate and ethanol accumulation. We also proposed that overexpression of genes for electron transfer systems, such as cytochrome c551 in *C. butyricum* and cytochrome c550, c551, b5 and electron transfer flavoprotein in *C. beijerinckii* enhanced electron transfer from the electrode and positively affected NADH pools in these bacteria (Fig. 6b, d, 7b, d).

Overall, our results suggest that the increased population of *C. butyricum* and *C. beijerinckii* upon bio-augmentation of the MC with *B. subtilis* and the presence of the electrical current enhanced electron transfer to the MC from the cathode, resulting in electron flow via NADH to generation of butyrate, ethanol and acetate, in both the ammonium- and nitrate-enriched wastewaters. Furthermore, electrical current stimulated the OE-NasDEF + MC system's capacity to assimilate ammonium from nitrate-enriched wastewater. In these systems we used 1% glucose to supplement cost-free sources of wastewater. This supplement can be made economically viable by recovery of high value fermentation products such as butyrate [67]. The economical feasibility of the system can be further improved by using carbon-rich wastewaters collected from e.g. the dairy industry [68,69]. The proposed method in this study yielded 0.35–0.39 g butyrate/g glucose consumed when toxic concentration of nitrogenous compounds decreased in effluent and partly recovered in anodic compartment. The highest yield of 0.45 g and 0.48 g butyrate/g glucose are produced using synthetic medium including glucose and yeast extract in extractive fermentation and repeated fed-batch in fibrous bed bioreactor, respectively [70,71]. Further optimization strategies such as bio-augmentation with different ratios of *B. subtilis* vs MC cells and improving the interaction of *Clostridium* with the solid electrode need to be explored to achieve higher butyrate yield from wastewater source. The discovery of DEGs involved in electron transfer from solid electrode could be used as leads to further engineer *Clostridium* strains with higher capacity of electron transfer, further reducing the electricity requirement for the

denitrification process. The proposed bio-augmented electrofermentation method in our BES shifted microbial metabolism towards nitrate reduction relevant for wastewater treatment, coupled to butyrate production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2020.125437>.

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