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Initial pH influences microbial communities composition in dark fermentation of scotta permeate

Ciro Vasmara [a], Massimo Pindo [b], Diego Micheletti [b], Rosa Marchetti [a,*]

[a] Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA), Research Centre for Animal Production and Aquaculture, Via Beccastecca 345, 41018, San Cesario Sul Panaro, Modena, Italy

[b] Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38010, San Michele all'Adige, Italy

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ABSTRACT

This study addresses for the first time the influence of initial pH on the evolution of microbial consortia in dark fermentation of scotta permeate, using a high-throughput sequencing approach. Three fermentation phases could be detected: 1) a lag phase with no substantial differences in microbial composition at different initial pH values; 2) an exponential H_2 production phase, accompanied by a general increase of *Clostridium* genus components and higher incidence of *Trichococcus* genus at neutral and alkaline pH; 3) a final stationary phase, characterized by a general increase of *Bifidobacterium* and *Lactobacillus* genera in all reactors. The initial pH value influenced the relative abundance of *Trichococcus* at 16–48 h of incubation. The metabolic activity of this genus increased the amount of metabolic precursors of H_2 so that, when pH lowered to 5.4, clostridia in the reactors with initial alkaline pH become more active H_2-producers than those in the others.

Introduction

In recent years, there has been a growing interest by the international scientific community towards biological hydrogen production [1]. The use of H_2 as an energy carrier is especially attractive because it has higher specific heat capacities (14.3 kJ kg^{-1} K^{-1}), in comparison with other currently used energy carriers, and because its combustion does not pollute the environment. In fact, the only by-product of H_2 combustion is water. Two fundamental ways of biological H_2 production are reported: a "light-dependent" H_2 production and a fermentative H_2 production, which is called *dark fermentation* (DF) because, in contrast with the other way of H_2 production, does not need any light [1]. The main DF agents belong to the genera *Enterobacter* and *Clostridium* [2–4]. Species from both of them are able to break up the carbohydrate molecules to pyruvate following the glycolytic pathway. However, they differ in the way of H_2 production from pyruvate. *Enterobacter* spp. convert the pyruvate molecule to Acetyl Co-A and formate by means of the enzyme pyruvate:formate lyase (Pfl), and then they use a

* *Corresponding author.*
E-mail address: rosa.marchetti@crea.gov.it (R. Marchetti).
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hydrogenase enzyme, Hyd3, to convert formate to H_2 and CO_2. The Hyd3 hydrogenase is peculiar of *Enterobacter* spp. Conversely, the *Clostridium* species transform pyruvate into Acetyl Co-A and CO_2. Reduced ferredoxin is then produced by pyruvate ferredoxin oxidoreductase (PFOR). Reduced ferredoxin feeds electrons directly to the [FeFe] hydrogenase, an enzyme typical of Clostridia, which transfers them to H_2^+ with H_2 formation [5].

The best substrates for H_2 production are those rich in carbohydrates, and cheese factory waste is particularly suitable for H_2 production because it contains large amounts of lactose [6–11]. Every year in Europe 40 million tons of cheese whey (CW) are produced: part of it is used mainly for animal feeding whereas the surplus production (13 million tons) can potentially supply 619 tons of lactose for H_2 production in DF [12]. In Italy, large amounts of cheese whey are used for the production of *ricotta*. Ricotta is a milk-derived product typical of the Mediterranean area. The ricotta production leaves a partially deproteinized CW, called *scotta*, having a high organic load [13]. In a previous paper we demonstrated the suitability of permeate from scotta for H_2 production, and the pH influence on the DF process [14]. Specifically, in this study, we measured the rate and yield of H_2 production from unbuffered permeate during 96 h in the pH range 4.0–10.0. pH evolution, lactose consumption, ethanol, lactic acid and volatile fatty acids production during DF were also monitored. On the basis of the pH trend and H_2 production, 3 phases could be detected: a lag phase (0–16 h, phase 1), an exponential H_2 production phase (16–48 h, phase 2) and a final stationary phase (72–96 h, phase 3). In the first 16 h of fermentation, a pH stabilization was observed in all the reactors. The mean pH value at the end of this period was equal to 5.4 (±0.1). In the time that followed, the pH course still decreased and was very uniform in all the reactors. The highest H_2 production occurred at pH = 8.7. Our results supported or integrated those reported by several authors [15–21].

The current study has been performed with the intention of addressing the effect of the initial pH value on the composition of microbial consortia in DF, and to better understand the relationship between metabolites production and DF agents. A high-throughput sequencing (HTS) approach was adopted since this increasingly widespread technology allows for a rapid response and a high coverage of the communities, with relatively low-costs [22]. The HTS technology has been recently used to study the microbiology of methanogenic reactors treating industrial wastewater [23,24], as well as to describe the microbial communities in hydrogen-producing reactors working under different conditions [25,26]. More recently, Etchebehere et al. [27] used this technique to compare the resident microbial communities in 20 hydrogen-producing reactors, located in different laboratories of South America.

The aim of this work was therefore to evaluate the influence of the initial pH value on the composition of the microbial communities involved in the DF process, using permeate of scotta as substrate. At 7 selected measurement times (0, 6, 16, 24, 48, 72 and 96 h) the microbial communities evolution in the reactors with initial pH values of 4, 8 and 10 was monitored using the HTS of the 16S rRNA genes.

Materials and methods

Feedstock

Liquid scotta permeate was used as raw material for DF. It had been obtained by ultrafiltration of scotta in the ENEA laboratory of Casaccia (Rome, Italy). Scotta was the by-product of the ricotta production by a large Italian dairy and cheese-making company. The scotta permeate was immediately stored at −28 °C until use. Information on permeate composition can be found in Ref. [14].

Experimental conditions

The experiment has been described in detail in Ref. [14]. Here we only resume basic information on DF conditions, and on the supplemental measurements, which were carried out on selected samples to study their microbial composition by metataxonomic analysis.

Dark fermentation

Dark fermentation at pH starting values ranging from 4 to 10, step 1, had been carried out in laboratory, throughout a 96-h period, at 35 °C, in strictly anaerobic conditions.

The DF reactors (500-mL volume) contained 200 mL permeate and 20 mL inoculum. The inoculum was produced in our laboratory using pig slurry as starting material and following the procedure described in Ref. [28].

Gas volume and composition, pH, and microbial metabolites (lactose, ethanol, volatile fatty acids and lactic acid) had been measured at 10 time intervals. Measurement methods are reported in Ref. [14]. For the purposes of this study 5-mL samples collected at 3 pH initial values (4, 8, and 10), and 6 time intervals (6, 16, 24, 48, 72 and 96 h) were selected as representative of the entire data set. Five-mL samples of fermentation broth were collected from each reactor for DNA extraction and molecular analysis, with 2 replicates for each treatment (36 samples in total). Two samples of the initial inoculum and of permeate were included in the analysis.

DNA extraction

Each 5-mL sample was centrifuged at 12400 relative centrifugal force for 1 min. A 2-mL pellet was recovered for each sample, and used for genomic DNA extraction. DNA was extracted using the PowerBiofilm® DNA Isolation Kit (Mo Bio laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

Library preparation and sequencing

The specific bacterial primer set 515F (5′ GTGYCAGCMGCCGCGGTAA 3′) and the 806R (5′ GGACTACNVGGGTWTCTAAT 3′) was used [29] with degenerate bases suggested by Ref. [30] with overhang Illumina adapters. Total genomic DNA was subjected to PCR amplification by targeting a ~250-bp fragment of the 16S rRNA variable region V4. Each sample was amplified by PCR using 25 μl reaction