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Short Communication

Proteomic profiling of an undefined microbial consortium cultured in fermented dairy manure: Methods development

The production of polyhydroxyalkanoates (PHA; bioplastics) from waste or surplus feedstocks using mixed microbial consortia (MMC) and aerobic dynamic feeding (ADF) is a growing field within mixed culture biotechnology. This study aimed to optimize a 2DE workflow to investigate the proteome dynamics of an MMC synthesizing PHA from fermented dairy manure. To mitigate the challenges posed to effective 2DE by this complex sample matrix, the bacterial biomass was purified using Accudenz gradient centrifugation (AGC) before protein extraction. The optimized 2DE method yielded high-quality gels suitable for quantitative comparative analysis and subsequent protein identification by LC-MS/MS. The optimized 2DE method could be adapted to other proteomic investigations involving MMC in complex organic or environmental matrices.

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2DE / Accudenz gradient centrifugation / Mixed culture biotechnology / Mixed microbial consortia
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The production of polyhydroxyalkanoates (PHA) using mixed microbial consortia (MMC) and fermented waste/surplus feedstocks has attracted attention as an inexpensive alternative to current commercial production which relies on pure cultures and refined substrates [1]. To be successful, the MMC must be highly enriched for PHA-producing bacteria. This is commonly achieved through aerobic dynamic feeding (ADF) [2], which imposes transient exogenous carbon availability (i.e., "feast-famine" conditions). However, advancement of this process has been impeded by an incomplete understanding of the physiology and biochemistry of the MMC response to ADF conditions. 2DE-based proteomics offers a powerful approach to elucidate functional responses; however, optimal 2DE procedures have not been established for MMC cultured in fermented waste feedstocks. To address this need, the research herein focused on the application of 2DE to an MMC subjected to ADF conditions and cultured on fermented dairy manure.

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Abbreviations: ADF, aerobic dynamic feeding; **AGC**, Accudenz gradient centrifugation; **MMC**, mixed microbial consortia; **PHA**, polyhydroxyalkanoates

Like other 2DE-based investigations involving mixed culture biotechnology (e.g., activated sludge processes and anaerobic digestion), the fermented dairy manure sample matrix added to the inherent complexity of MMC protein mixtures. Direct protein extraction from MMC biomass and subsequent electrophoretic separation is challenging due to the abundance of non-bacterial protein impurities and non-bacterial solids (including volatile fatty acids, salts, crude fat, non-structural carbohydrates, inert particulates, lignocellulose degradation products, and pigmented compounds).

To combat similar interferences, indirect protein extraction approaches utilizing gradient centrifugation that first isolate/purify bacterial cells from complex matrices have been applied in MMC proteome studies of soil and freshwater samples using 1D SDS PAGE [3, 4]. Drawing from these investigations, the aim of this study was to optimize an MMC sample processing procedure using Accudenz gradient centrifugation (AGC) and sequential protein extraction to improve the recovery of bacterial proteins from reactor biomass and ensure effective protein separation by 2DE.

Sample source: MMC biomass samples were obtained from a sequencing batch reactor operated under ADF conditions conducive for PHA synthesis. Briefly, the reactor was fully aerobic, constantly mixed, had a volume of 1.8 L, solids and hydraulic retention time of 4 d, and cycle length of 24 h.

Colour Online: See the article online to view Figs. 1-3 in colour

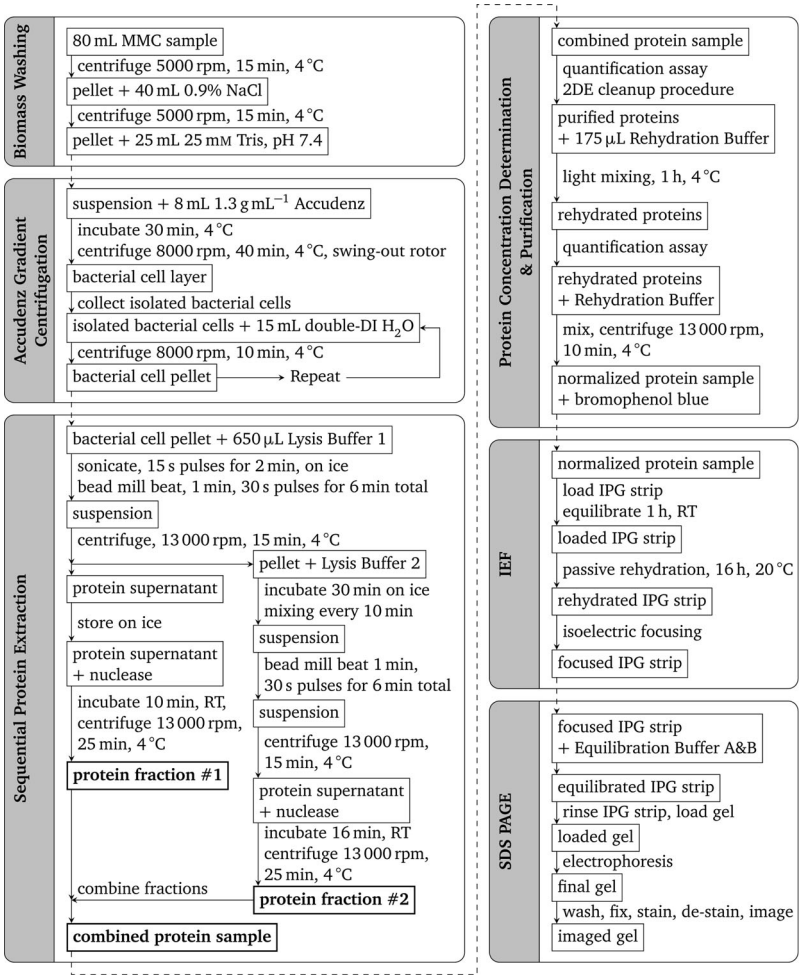

Figure 1. Outline of the optimized "AGC 2DE" workflow. See text for additional details.

The fermented dairy manure used for substrate was prepared in the following manner: effluent from a lab-scale manure fermenter was screened to remove large solids, centrifuged at 8000 rpm for 5 min to remove fine solids, autoclaved, and diluted with tap water prior to addition to the reactor. The suspended solids concentration (including microbial biomass) in the reactor was maintained above 2000 mg/L.

Optimized "AGC 2DE" method: The following procedures are outlined in Fig.1. All reagents used were of electrophoresis-grade. Initial biomass collection and washing was adapted from Wilmes and Bond [5] with minor modifications. Briefly, 80 mL MMC sample was centrifuged at 5000 rpm for 15 min at 4°C in an SS-34 rotor (Sorvall, Waltham, MA). The supernatant was discarded; the pellet was resuspended in 40 mL of 0.9% NaCl and washed by centrifugation at 5000 rpm for 15 min at 4°C. After discarding the wash, the pellet was resuspended in 25 mL of 25 mM Tris, pH 7.4.

The AGC procedures were adapted from published protocols [3, 6] with modifications. Briefly, to the 25 mL washed biomass suspension, 8 mL of 1.3 g/mL Accudenz (Accurate Chemical Company, Westbury, NY) solution was added beneath the sample and incubated for 30 min at 4°C. The sample was centrifuged at 8000 rpm for 40 min at 4°C in a JS13.1 swing-out rotor (Beckman Coulter, Pasadena, CA). Separated bacterial cells at the gradient interface (a visible turbid layer) were collected with a 5 mL syringe fitted with an 18-gauge needle bent at 90° (this size permits bacterial cell flow). Collected cells were washed twice in 15 mL of double-DI H_2O by centrifugation at 8000 rpm for 10 min at 4°C using the same rotor; washes were discarded and the bacterial cell pellet was subjected to sequential protein extraction.

For sequential protein extraction, the bacterial cell pellet was resuspended in 650 µL Lysis Buffer 1 (25 mM Tris pH 7.4, 0.1 mM EDTA, and 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)), vigorously mixed, and transferred to a 1.5 mL self-standing screw cap tube. The bacterial cell suspension was sonicated using a Model 100 Sonic Dismembrator (Thermo Fisher, Waltham, MA) on ice with 15 s pulses up to 2 min with the power setting at 3, after which 0.1 mm zirconia/silica beads (Biospec Products Inc., Bartlesville, OK) were added to the suspension to approximately half the sample volume. The suspension was homogenized in a bead mill beater (Biospec Products Inc.) for 1 min, followed by 30 s pulses for 6 min, and then centrifuged at 13 000 rpm for 15 min at 4°C, and the supernatant (protein

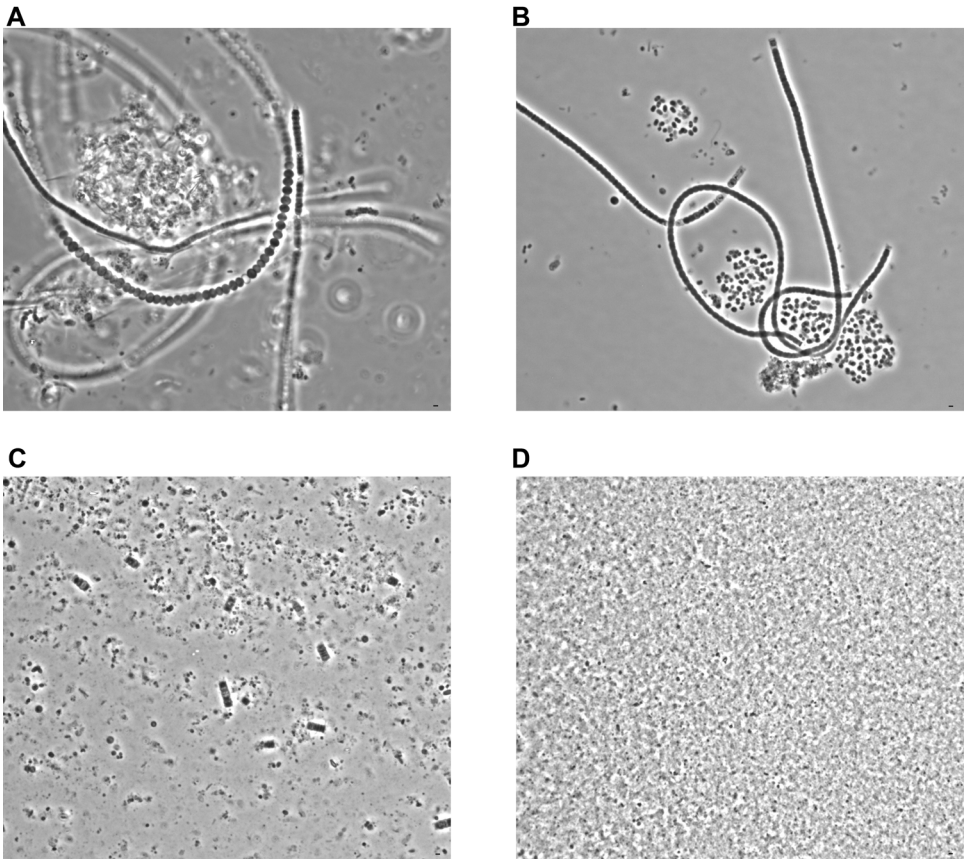

Figure 2. Bacterial cell separation and disruption. Phase contrast images (1000x) showing representative images of (A) biomass directly from the MMC reactor, (B) bacterial cells isolated using AGC, (C) bacterial suspension after brief sonication and first bead mill beating in Lysis Buffer 1, and (D) bacterial suspension after 30 min incubation and second bead mill beating in Lysis Buffer 2. Images were acquired using a Nikon Eclipse 55i phase contrast microscope with NIS-147 Elements Br. 3.0. Bars in the bottom right corner represent 1 μm.

fraction #1) was stored on ice. The pellet was resuspended in 600 μL Lysis Buffer 2 (7 M urea, 2 M thiourea, 4% w/v CHAPS, 10 mM Tris-1 mM EDTA, 50 mM DTT, and 0.5 mM AEBSF; DTT, CHAPS and AEBSF were added immediately before use), then incubated on ice for 30 min with vigorous mixing every 10 min. The aforementioned bead mill beating procedure was repeated, along with centrifugation and supernatant retention (protein fraction #2). A nuclease treatment was performed separately on each protein fraction by adding 50U of Benzonase nuclease (Sigma-Aldrich, St. Louis, MO) and incubating at room temperature for 10 min with light mixing every 2 min. Suspensions were centrifuged at 13 000 rpm for 25 min at 4°C; the supernatants were combined into one sample and vigorously mixed. When required, protein samples were stored at –80°C.

Protein concentration of the combined fractions was determined by the RCDC Protein Assay (Bio-Rad, Hercules, CA) following the manufacturer's instructions and using BSA as the protein standard. Protein precipitation and clean-up procedures were performed using a ReadyPrep 2-D Cleanup Kit (Bio-Rad) following the manufacturer's instructions. The protein pellet was resuspended in 175 μL Rehydration Buffer (7 M urea, 2 M thiourea, 3% (w/v) CHAPS, 50 mM DTT, and 0.2% (w/v) Bio-Lyte 3/10 ampholytes; DTT, CHAPS, and ampholytes were added immediately before use). The sample was mixed for 1 h at 4°C. Protein concentration in the rehydration solution was determined using the RCDC Assay, and the sample was diluted in Rehydration Buffer to normalize the concentration to 3.2 mg/mL; a trace amount of bromophenol blue was added, followed by vigorous mixing and centrifugation at 13 000 rpm for 10 min at 4°C prior to IPG strip loading.

For IEF, 400 μg protein in 125 μL was loaded onto a 7 cm ReadyStrip IPG Strip pH 4–7 (Bio-Rad), equilibrated for 1 h at room temperature, covered with mineral oil, and passively rehydrated for 16 h at 20°C. The IPG strip was focused in a PROTEAN IEF Cell (Bio-Rad) at 250 V for 15 min, followed by linear ramping to 4000 V in 2.5 h, after which 4,000 V was held for approximately 3.25 h (for a total of 13,000 Vh). Immediately following IEF, the IPG strip was equilibrated twice for 20 min each in Equilibration Buffer A (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris pH 8.8, and 2% (w/v) DTT) and Equilibration Buffer B (same as A only DTT was replaced by 2.5% (w/v) iodoacedamide) with gentle shaking; for each equilibration, fresh buffer was exchanged after 10 min.

For SDS PAGE separation, the IPG strip was rinsed in Tris-Glycine-SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), loaded onto an 8 × 10 cm 12% Mini-PROTEAN TGX pre-cast gel (Bio-Rad), sealed with overlay agarose, and electrophoresed at 70 V for 40 min, followed by 150 V until the tracking dye reached the bottom of the gel; molecular weight markers were applied to an electrode wick and inserted into the gel before the application of overlay agarose.

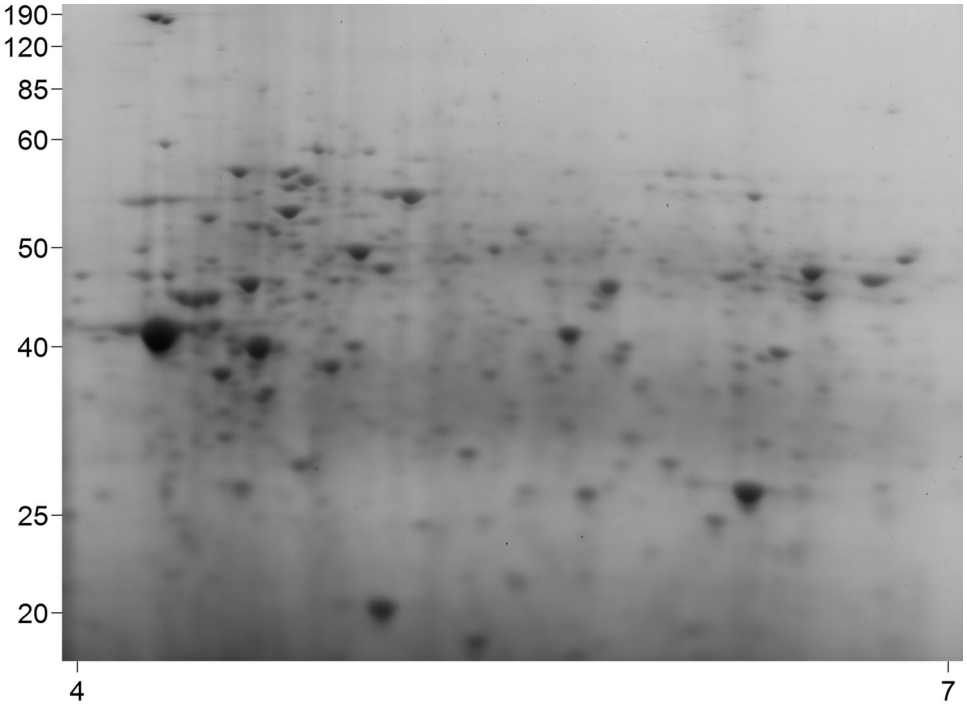

Figure 3. Representative 2DE gel image for the optimized "AGC 2DE" method. Molecular weight markers (in kDa) are listed to the left of the image, and the pH range of the IPG strip is depicted below the image.

The gel was washed twice in double-DI H_2O for 5 min, fixed in ethanol/acetic acid/water (40:10:50 (v/v)) for 30 min, stained with pre-made Coomassie Brilliant Blue G-250 (CBB; Bio-Rad) for 20 h, and de-stained with double-DI H_2O for several hours. The gel was scanned using an Odyssey Imaging System (Li-COR Biosciences, Lincoln, NE) with the following settings which generated 16 bit, 600 DPI TIFF files: resolution, 42 μm; quality, medium; focus offset, 0.5; detection channel, 700 nm. REDFIN 3 Gel Image Analysis Software (Ludesi, Malmö, Sweden) was used for gel image analysis following the manufacturer's recommendations related to image quality control, gel warping, spot matching, spot intensity measurement, background correction, and spot volume normalization.

The primary objective during the development of the "AGC 2DE" workflow (Fig. 1) was to increase the reliability and reproducibility of 2DE for investigating MMC operated under ADF conditions. Initial 2DE attempts indicated significant interference from the sample matrix and biased protein recovery, in addition to poor-quality gels (data not shown). AGC and sequential protein extraction were incorporated into the method to address these shortcomings.

AGC was used to mitigate the interference from the fermented dairy manure matrix on the bacterial protein extraction by separating the bacterial cells from the non-bacterial solids. While AGC may introduce bias into the bacterial recovery [7], priority was given to achieving successful 2DE. To that end, AGC removed the non-bacterial solids from the sample, reducing the potential for the co-extraction of impurities and partitioning of proteins during extraction. The suspended solids concentration in the MMC reactor was 2394 ± 736 mg/L, and as shown in Fig. 2A and B, AGC effectively isolated bacterial cells from other solids. The bacterial cell pellet was typically 1/3 of the initial biomass pellet wet mass, reinforcing the value of AGC as a purification tool to separate bacterial cells from matrix debris.

Sequential protein extraction was adopted in an effort to maximize bacterial protein recovery while reducing extraction bias. As shown in Fig. 2B, cell morphology was diverse in the MMC; the brief sonication, coupled with two rounds of bead mill beating applied in the "AGC 2DE" method aided cell disruption (Fig. 2C and D) to help maximize protein recovery. No protein loss was observed following protein fraction combination, allaying concerns that protein precipitation might occur due to buffer dissimilarity. Throughout "AGC 2DE" development, the protein concentration in the combined protein sample was 12.8 ± 5.5 mg/mL, which was sufficiently high for the 2DE clean-up and purification procedures. Together with the identification of both cytoplasmic and hydrophobic membrane-bound proteins (excised from the 2DE gel and identified by LC-MS/MS using the procedures adapted from Checinska et al. [8]; data not shown), the effectiveness of the sequential protein extraction was reinforced.

The "AGC 2DE" method was repeated multiple times and consistently yielded gels with spot patterns similar to that shown in Fig. 3. Throughout "AGC 2DE" optimization, the number of detected protein spots ranged from 585 to 639, with an average of 608 ± 26; spot numbers for the "AGC 2DE" method were higher compared to 2DE attempts involving direct extraction (data not shown). Other positive results of the "AGC 2DE" method included well-resolved spots, minimal horizontal streaking, reduced vertical stacking, and nominal background interference (Fig. 3). Proteins from the "AGC 2DE" method exhibited an array of isoelectric

points across the pH 4 to 7 range, and an apparent molecular weight range spanning from less than 15 kDa up to 190 kDa. IPG strips with pH 3–10 and 7–10 ranges were evaluated, in addition to active IPG strip rehydration; however, protein resolution was not improved (data not shown). CBB G-250 was ultimately chosen over other evaluated stains (CBB R-250, Silver Stain for MS, and SYPRO Ruby) for its low background interference, reproducibility, sensitivity when used in combination with infrared scanning, and compatibility with LC-MS/MS.

The high-quality 2DE gels resulting from the "AGC 2DE" method enabled protein spot identification via LC-MS/MS, which complimented quantitative comparative assessments. As an example, LC-MS/MS analysis of one protein spot and subsequent MS/MS ions search using MASCOT (Matrix Science) resulted in the assignment of four proteins to *Meganema perideroedes* (the MASCOT search criteria used and corresponding protein identification information is provided in Supporting Information Tables S1–S3). The highest scoring protein was an amino acid ABC transporter substrate-binding protein with 17 peptide matches representing 74% amino acid sequence coverage. The other candidate proteins associated with the excised protein spot included two hypothetical proteins and LacI transcriptional regulator, each assigned to *M. perideroedes*. The MS proteomics data have been deposited to the ProteomeXchange Consortium [9] via the PRIDE partner repository with the dataset identifier PXD003004 and 10.6019/PXD003004; the MASCOT result file, peak list file, and all raw LC-MS/MS data can be accessed through the PRIDE repository.

The optimized "AGC 2DE" procedures presented herein were effective as a biomass processing method for MMC protein profiling in an engineered system laden with 2DE-interfering impurities and non-bacterial solids. The "AGC 2DE" method yielded reproducible, high-quality 2DE gels from which protein spots have been excised and identified via LC-MS/MS. Notably, the presented workflow could be modified based on available laboratory equipment; for example, a similar make or model of swing-out rotor could be substituted, different cell disruption methods could be implemented, or alternative gel imaging systems could be used in place of an infrared scanner. As such, the "AGC 2DE" workflow could be adapted to other MMC in complex matrices often encountered in mixed culture biotechnology studies as part of a gel-based or gel-free proteomics approach.

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The authors declare no conflicts of interest.

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