Metabolome Analysis of Constituents in Membrane Vesicles for Clostridium thermocellum Growth Stimulation

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Abstract: The cultivation of the cellulolytic bacterium, Clostridium thermocellum, can have cost-effective cellulose biomass utilizations, such as consolidated bioprocessing, simultaneous biological enzyme production and saccharification. However, these processes require a longer cultivation term of approximately 1 week. We demonstrate that constituents of the C. thermocellum membrane vesicle fraction significantly promoted the growth rate of C. thermocellum. Similarly, cell-free Bacillus subtilis broth was able to increase C. thermocellum growth rate, while several B. subtilis single-gene deletion mutants, e.g., yxeJ, yxeH, abpC, yxdK, iolF, decreased the growth stimulation ability. Metabolome analysis revealed signal compounds for cell–cell communication in the C. thermocellum membrane vesicle fraction (ethyl 2-decenoate, ethyl 4-decenoate, and 2-dodecenoic acid) and B. subtilis broth (nicotinamide, indole-3-carboxaldehyde, urocanic acid, nopaline, and 6-paradol). These findings suggest that the constituents in membrane vesicles from C. thermocellum and B. subtilis could promote C. thermocellum growth, leading to improved efficiency of cellulose biomass utilization.

Keywords: cellulose biomass utilization; membrane vesicle; cell–cell communication; Clostridium thermocellum; Bacillus subtilis; metabolome analysis

1. Introduction

Cellulose is one of the most abundant organic materials on Earth. Bacteria that can grow on cellulose have been isolated from many environments that include soil, hot springs, cow rumen, termite gut, and the human intestinal tract [1]. Clostridium thermocellum (Acetivibrio thermocellus) [2], a Gram-positive thermophilic anaerobic soil bacterium, is a candidate for cellulose biomass utilization. C. thermocellum completely degrades 4.4 g/L purified cellulose in one day [3]. It also degrades 65% of 5 g/L switchgrass in five days and 70% of 10 g/L corn hull in seven days [4,5].

C. thermocellum has been shown to produce 1.3% ethanol from 10% Avicel cellulose [6]. A strain of C. thermocellum multiply deleted for [FeFe] hydrogenase maturase, lactate dehydrogenase, pyruvate-formate lyase, Pfl-activating enzyme, phosphotransacetylase, and acetate kinase genes, which eliminated formate, acetate, and lactate production, and reduced H2 production, presented a titer of 2.2% ethanol from 6% Avicel cellulose [7]. The ethanol hyper-producing strain C. thermocellum I-1-B produced 2.4% ethanol from 8% cellulose [8]. A co-culture of a strain lacking the lactate dehydrogenase/phosphotransacetylase gene and Thermostreptococcus thermophilus produced 3.8% ethanol from 9.2% Avicel cellulose in 146 h [9]. These reports show that the cultivation of C. thermocellum can be simplified consolidated bioprocessing (CBP). This is a promising strategy because it eliminates the need to add lignocellulose-degrading enzymes that significantly increase the cost of biofuel production [10–12].
Some cellulolytic bacteria, including \textit{C. thermocellum}, form carbohydrate-active enzyme (CAZyme) complexes that are termed cellulosomes \cite{13–16}. The main product of enzymatic cellulose degradation is cellobiose, which leads to the feedback inhibition of cellulosomes. Supplementation with \(\beta\)-glucosidase (BGL) leads to the hydrolysis of cellobiose into two glucose molecules, thereby resolving the feedback inhibition. \textit{C. thermocellum} preferentially utilizes cellooligosaccharide, and glucose tends to accumulate in the culture broth \cite{17}. Supplementation with purified BGL increased glucose production by \textit{C. thermocellum} from 10\% cellulose or 12\% alkali pretreated rice straw by approximately 7.7\% over 10 days \cite{18}. This technology is referred to as biological simultaneous enzyme production and saccharification (BSES). BSES is similar to CBP, does not require the diverse CAZymes for the saccharification of cellulosic biomass.

We previously reported that \textit{C. thermocellum} produces extracellular membrane vesicles (MVs) that are released into the broth \cite{19}. MVs are produced in Gram-negative and Gram-positive bacteria. The latter possess a membrane that is overlaid by a relatively thick and resilient cell wall enriched in peptidoglycan \cite{20,21}. MVs have been isolated from the culture supernatant of Gram-positive bacteria that include \textit{Bacillus subtilis}, \textit{B. anthracis}, \textit{Streptomyces coelicolor}, \textit{Listeria monocytogenes}, \textit{Staphylococcus aureus}, \textit{Streptococcus mutans}, \textit{S. pneumoniae}, and \textit{Clostridium perfringens} \cite{22–28}. Klieve et al. reported the production of MVs by \textit{Ruminococcus} spp., a cellulolytic bacterium that resides in the ovine rumen. DNA molecules ranging in size from <20 to 49 kb, and from 23 to 90 kb are attached to MVs from \textit{Ruminococcus} sp. YE73 and \textit{Ruminococcus albus} AR67, respectively. Thus, MVs can function as vectors for horizontal gene transfer to confer cellulolytic activity, as documented in the mutant strain \textit{Ruminococcus} sp. YE71 \cite{29}. MVs from cellulolytic \textit{Bacteroides fragilis} and \textit{B. thetaiotaomicron} are equipped with hydrolytic enzymes and are important in polysaccharide degradation \cite{30,31}. MVs from \textit{Fibrobacter succinogenes} are enriched with CAZymes, and intact MVs are able to degrade a broad range of hemicelluloses and pectin \cite{32}. We have previously proposed that \textit{C. theromboellum} may utilize MVs to deliver cellulosomes, which enhance the cellulolytic activity of \textit{C. thermocellum} \cite{19}.

MVs contain various compounds that include DNA and RNA. These cargos are delivered to neighboring cells. MVs have several important functions related to cell–cell interactions. In \textit{Pseudomonas aeruginosa}, a hydrophobic cell–cell communication signal termed \textit{Pseudomonas} quinolone signal is released from the bacteria via MVs \cite{33,34}. MVs can also serve as organic carbon sources for heterotrophs. For example, MVs derived from cyanobacteria support the growth of \textit{Alteromonas} and \textit{Halomonas} as the sole carbon source, indicating that MVs should be considered in the marine food web and may have important roles in the carbon flux of the ocean \cite{35}. In \textit{Mycobacterium tuberculosis}, the causative agent of tuberculosis, increased MV production in response to iron restriction has been observed \cite{36}. These MVs contain a siderophore called mycobactin. Mycobactin can serve as an iron donor to support the growth of iron-starved \textit{M. tuberculosis}.

In this study, we demonstrated that the MV fractions collected from \textit{C. thermocellum} and \textit{B. subtilis} can promote \textit{C. thermocellum} growth. Metabolome analysis was also performed to identify the candidate compounds with the growth stimulation.

2. Materials and Methods

2.1. Strains and Culture Conditions of \textit{C. thermocellum} and \textit{B. subtilis}

One hundred microliters of \textit{C. thermocellum} DSM 1313 (DSMZ, Braunschweig, Germany) culture was inoculated in 5 mL of CTFUD medium (3 g/L sodium citrate tribasic dehydrate, 1.3 g/L \(\text{(NH}_4\text{)}_2\text{SO}_4\), 1.5 g/L \text{KH}_2\text{PO}_4, 130 mg/L \text{CaCl}_2 \cdot 2\text{H}_2\text{O}, 500 mg/L \text{L-cysteine-HCl}, 11.56 g/L 3-morpholinopropanesulfonic acid, 2.6 g/L \text{MgCl}_2 \cdot 6\text{H}_2\text{O}, 1 mg/L \text{FeSO}_4 \cdot 7\text{H}_2\text{O}, 4.5 g/L \text{Bacto yeast extract}, 1 mg/L \text{resazurin}, \text{pH} \text{7.0}) containing 0.5\% cellobiose (Tokyo Chemical Industry, Tokyo, Japan) with 16 \times 125 mm Hungate tubes (Chemiglass Life Sciences, Vineland, NJ, USA), and cultured at 60 °C under anaerobic conditions with nitrogen gas \cite{37}.
B. subtilis KAO/NAIST chromosomal deletion mutants [38] and BKE genome-scale deletion mutants [39] were obtained from the National BioResource Project B. subtilis (National Institute of Genetics, Shizuoka, Japan). B. subtilis strains were aerobically cultured in Luria Bertani broth at 37 °C.

2.2. Preparation of MV Fraction of C. thermocellum

Five milliliters of C. thermocellum and B. subtilis culture was centrifuged at 10,000×g for 2 min at 4 °C, and the supernatant was filtered through a 0.22-µm syringe filter to remove cells. The filtrate was centrifuged at 179,000×g for 1 h at 4 °C and the pellet was washed twice with 2 mL of sterile phosphate-buffered saline (PBS). The pellet was resuspended in PBS and used as the MV fraction. The MV fraction was kept on ice before use.

MVs were visualized using transmission electron microscopy. Six microliter aliquots of the MV fraction was added to 300-mesh carbon and formvar-coated copper grids and incubated for 1 min. After removing the extra solution with filter paper, each specimen was stained with 2% phosphotungstic acid. The sample was observed with a JEM-1011 microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.3. Growth Evaluation of C. thermocellum with MV Supplementation

One hundred microliters of C. thermocellum DSM 1313 culture was inoculated in 5 mL of CTFUD medium containing 0.5% cellobiose with the supplementation of the collected MV fraction. C. thermocellum was cultured at 60 °C under anaerobic conditions with nitrogen gas. The C. thermocellum growth was evaluated with optical density of the broth at 600 nm.

2.4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis of C. thermocellum MV and B. subtilis Broth

The C. thermocellum MV fraction was treated with 10 mg/L surfactin, and the filtrate obtained after ultrafiltration with Vivaspin 2-100 K (Cytiva, Marlborough, MA, USA) was used to obtain the constituents in MVs. Cell-free supernatants of B. subtilis trpC2 and trpC2 yxeJ broth were prepared by centrifugation and filtration with a 0.22-µm syringe filter. These specimens were homogenized with zirconia beads in 75% methanol, and the supernatants were collected after centrifugation at 15,000×g rpm for 10 min. The supernatants were applied to a MonoSpin C18 column (GL Science, Tokyo, Japan) and were filtered through a 0.22-µm syringe filter.

LC-MS analysis was performed on an Ultimate 3000 rapid separation LC (RSLC) and the Q Exactive system (Thermo Fisher Scientific, Waltham, MA, USA). Ultimate 3000 RSLC analysis was performed with the following parameters: column, InertSustain AQ-C18 (GL Science); column temperature, 40 °C; injection volume, 2 µL; solvent flow rate, 200 µL/min. The eluting solution was 0.1% formic acid containing 2% acetonitrile. The Q Exactive system had the following parameters: measurement time, 3–30 min; ionization method, electrospray ionization; measurement mass range, m/z: 80–1200; full scan resolution, 70,000; and MS/MS scan resolution, 17,500. The obtained data were analyzed with PowerGetBatch and MFSearcher [40]. The LC-MS analysis was performed in triplicate.

3. Results and Discussion

3.1. MV Constituents Promote C. thermocellum Growth

A previous study reported that the co-culture of the engineered C. thermocellum and T. saccharolyticum strains produced 3.8% ethanol from cellulose for 6 days [9]. C. thermocellum cultivation with BGL supplementation for 10 days reportedly produced 76.7 g/L glucose from alkali pretreated rice straw [18]. It seems that the growth rate of C. thermocellum is an important factor in improving the efficiency of CBP and BSES. In this study, we collected MVs from C. thermocellum broth (Figure S1). MVs contain various compounds, such as DNA and RNA, which function in cell–cell communication. When C. thermocellum was grown in the presence of the MV fraction, the growth rate did not change.
However, when the MVs were lysed using the lipopeptide surfactin [41] the cell density of *C. thermocellum* had significantly increased at 24 h after the inoculation (Figure 1). The surfactin supplementation alone did not affect the *C. thermocellum* growth rate. The final growth yield in each sample had not changed significantly. These results suggest that the constituents in the MV fraction could promote the growth rate of *C. thermocellum*.

![Figure 1](image-url)  
**Figure 1.** *C. thermocellum* growth stimulation by the MV constituents. *C. thermocellum* was cultured in CTFUD medium for 24 h with the supplementation of water, the MV fraction, or the surfactin-supplementation alone did not affect the *C. thermocellum* growth rate (Figure 2a). Mukamolova et al. purified the resuscitation promoting factor (Rpf) from the broth of the Gram-positive bacterium, *Micrococcus luteus*. The purified Rpf promoted the growth of this bacterium as well as *Mycobacterium avium*, *M. bovis*, *M. kansasii*, *M. smegmatis*, and *M. tuberculosis* [42]. Genes homologous to the *rpf* gene were found to be widespread in a number of *Mycobacterium* species, as well as in Gram-positive bacteria with a high GC content, such as *Corynebacterium gultamicum* and *Streptomyces rimosus*. The Rpf protein shows peptidoglycan degradation activity [43]. Shah et al. reported that muropeptide fragments released from the peptidoglycan of the Gram-positive bacterium, *B. subtilis*, stimulate the germination of bacterial spores. Stauroporine, which inhibits related eukaryotic kinases in bacteria, blocks muropeptide-dependent bacterial spore germination [44]. We evaluated the effect of stauroporine on *C. thermocellum* growth with cell-free *B. subtilis* broth, however no significant inhibition was observed.

We further evaluated the *C. thermocellum* growth promotion effect of the broth of *B. subtilis* genome deletion mutants [38]. All the mutants, especially six mutants in which the *pdp-rocR* genomic region, were deleted (MGB723, MGB773, MGB822, MGB834, MGB860, MGB874) promoted *C. thermocellum* growth by accelerating the growth rate (Figure 2b, Table S1). Subsequently, we evaluated the *C. thermocellum* growth promotion effect of 100 *B. subtilis* mutants in which single genes within the *pdp-rocR* genomic region were deleted under a *trpC2* gene deletion background (Table S2) [39]. We did not find *B. subtilis* mutants that promoted *C. thermocellum* growth more than *trpC2* strain as the parent strain. Contrary to our expectation, the effect of 23 *B. subtilis* mutants was significantly lower than that of the parent strain (Figure 2c).
Among these 23 genes, the functions of several genes have been experimentally evaluated. The *asnH* operon, which comprises *yxbB, yxbA, yxbN, asnH*, and *yxaM*, might be involved in the biosynthesis of asparagine [45]. The *iolJ, iolG, iolE, iolC, iolB*, and *iolR* genes in the *iolABCDEFGHII* and *iolRS* operon are responsible for *myo*-inositol catabolism involving multiple and stepwise reactions [46–48]. We observed a slight growth inhibition of *C. thermocellum* in the presence of *myo*-inositol, however this required a high concentration (1 mg/mL) of *myo*-inositol (Figure S2). YydF is predicted to be an exported and modified peptide that has antimicrobial and/or signaling properties [49,50]. YxaL, which contains a repeated pyrrolo-quinoline quinone (PQQ) domain that forms a beta-propeller structure, interacts with the DNA helicase PcrA in *B. subtilis* [51]. Kim et al. reported that treatment of *Arabidopsis thaliana* and *Oryza sativa* L. seeds with 1 mg/L purified YxaL was effective in improving root growth [52]. PQQ, which was first recognized as an enzyme cofactor in bacteria, displays bioactivities for various eukaryotes and prokaryotes. For many bacterial species, PQQ has growth stimulation effect and serves as a cofactor for a special class of dehydrogenases/oxidoreductases [53]. PQQ has been described as an essential growth factor for various microbes [54–56]. We observed a slight *C. thermocellum* growth promotion effect by PQQ. This effect was not enough to explain the effect of *B. subtilis* broth (Figure S3). More than 50 proteins are involved in *B. subtilis* spore coat assembly. Of these, YxeE is an inner spore coat protein [57,58]. *ahlpC* encodes thiol-specific peroxidase that plays a role in protecting cells against oxidative stress by detoxifying peroxides [59]. Utilization of a hydroxamate siderophore, ferrioxamine, requires the FhuBGC ABC transporter together with a ferrioxamine-binding protein, YxeB [60]. A range of siderophores can act
as growth factors for various previously uncultured bacteria [61]. YxdK is assumed to be a subunit of the two-component sensor histidine kinase, with its potential cognate response regulator, YxdJ [62]. Co-cultivation with *B. subtilis* allows the growth of *Synechococcus leopoliensis* CCAP1405/1 on solid media. However, the *yxdK* deletion mutant reportedly loses this ability [63]. The *yxeK* gene, which encodes FAD-dependent monooxygenase, contributes to the metabolism of S-(2-succino)cysteine to cysteine [64].

3.3. Metabolome Analysis of the Constituents in *C. thermocellum* MV and *B. subtilis* Broth

We collected the constituents in *C. thermocellum* MVs and analyzed them using LC-MS/MS. Among the 534 detected peaks, the intensities of seven peaks were significantly higher in the fraction where MVs had been disrupted by surfactin compared to MVs not disrupted using surfactin (Table S3). The structure of five significantly detected compounds in surfactin-treated *C. thermocellum* MVs specimen can be estimated by MS/MS analysis (Table 1 and Table S5).

**Table 1.** The constituents in *C. thermocellum* MVs detected by LC-MS/MS analysis.

| No.  | Formula   | Exact Mass | Name                     | Database | Database ID          |
|------|-----------|------------|--------------------------|----------|----------------------|
| 3203 | C₁₆H₃₁O₅N₁ | 253.241    | EX-HR2                   |          |                      |
| 3013 | C₁₂H₂₂O₂   | 198.162    | Ethyl 2-decenoate         | UC2      | HMDB0037329          |
|      | C₁₂H₂₂O₂   | 198.162    | Ethyl 4-decenoate         | UC2      | HMDB0039220          |
|      | C₁₂H₂₂O₂   | 198.162    | Methyl 9-undecenoate      | UC2      | HMDB0037305          |
|      | C₁₂H₂₂O₂   | 198.162    | Methyl 10-undecenoate     | UC2      | HMDB0029585          |
|      | C₁₂H₂₂O₂   | 198.162    | Allyl nonanoate           | UC2      | HMDB0029763          |
|      | C₁₂H₂₂O₂   | 198.162    | cis-3-Hexenyl hexanoate   | UC2      | HMDB0033378          |
|      | C₁₂H₂₂O₂   | 198.162    | 2-Hexenyl hexanoate       | UC2      | HMDB0038924          |
|      | C₁₂H₂₂O₂   | 198.162    | Hexyl 2E-hexenoate        | UC2      | HMDB0038269          |
|      | C₁₂H₂₂O₂   | 198.162    | Hexyl 2-methyl-3-pentenoate | UC2    | HMDB0040158         |
|      | C₁₂H₂₂O₂   | 198.162    | Hexyl 2-methyl-4-pentenoate | UC2    | HMDB0040163         |
|      | C₁₂H₂₂O₂   | 198.162    | 1-Ethenylhexyl butanoate  | UC2      | HMDB0037498          |
|      | C₁₂H₂₂O₂   | 198.162    | 2-Octenyl butyrate        | UC2      | HMDB0038081          |
|      | C₁₂H₂₂O₂   | 198.162    | cis-4-Decenyl acetate     | UC2      | HMDB0032214          |
|      | C₁₂H₂₂O₂   | 198.162    | Menthyl acetate           | UC2      | C0036314             |
|      | C₁₂H₂₂O₂   | 198.162    | Rhodinyl acetate          | UC2      | HMDB0037186          |
|      | C₁₂H₂₂O₂   | 198.162    | Citronellyl acetate       | UC2      | C0035564             |
|      | C₁₂H₂₂O₂   | 198.162    | 2-Dodecenoic acid         | UC2      | HMDB0010729          |
|      | C₁₂H₂₂O₂   | 198.162    | 4-dodecenoic acid         | UC2      | C0051284             |
|      | C₁₂H₂₂O₂   | 198.162    | 5-dodecenoic acid         | UC2      | HMDB0000529          |
|      | C₁₂H₂₂O₂   | 198.162    | 11-Dodecenoic acid        | UC2      | HMDB0032248          |
|      | C₁₂H₂₂O₂   | 198.162    | 5-dodecalactone           | UC2      | HMDB0037742          |
|      | C₁₂H₂₂O₂   | 198.162    | gamma-Dodecalactone       | UC2      | C0030347             |
|      | C₁₂H₂₂O₂   | 198.162    | epsilon-Dodecalactone     | UC2      | HMDB0038895          |
|      | C₁₂H₂₂O₂   | 198.162    | alpha-Heptyl-gamma-valerolactone | UC2  | HMDB0037813         |
|      | C₁₂H₂₂O₂   | 198.162    | 4-butyl-4-hydroxyoctanoic acid lactone | UC2  | HMDB0036182         |
|      | C₁₂H₂₂O₂   | 198.162    | 2,6-Dimethyl-5-heptenal propyleneglycol acetal | UC2  | HMDB0032235         |
|      | C₁₂H₂₂O₂   | 198.162    | citral dimethyl acetal    | UC2      | HMDB0040361          |
An aliphatic compound with the chemical formula C$_{12}$H$_{22}$O$_2$ was specifically detected in surfactin-treated C. thermocellum MVs (Table 1). Cis-2-decenoic acid was reported to decrease persister formation and revert dormant cells to a metabolically active state. Wang et al. demonstrated that three medium-chain unsaturated fatty acid ethyl esters (ethyl trans-2-decenoate, ethyl trans-2-octenoate, and ethyl cis-4-decenoate) decreased persister formation in Escherichia coli, P. aeruginosa, and Serratia marcescens, suggesting that fatty acid ethyl esters disrupt bacterial dormancy [65].

Some aliphatic acids function as diffusible signal factors (DSFs). These include cis-11-methyl-2-dodecenoic acid from Xanthomonas campestris and cis-2-dodecenoic acid from Burkholderia cenocepacia, among others [66]. DSFs are synthesized by and interact with a diverse group of microbes, including fungi, suggesting a broad conservation of cell-cell communication among these organisms [67–70]. Mutation of the DSF biosynthesis gene in B. cenocepacia results in substantially impaired growth in minimal medium [71]. Dean et al. demonstrated that Burkholderia DSF inhibits the formation and disperses Francisella biofilms. Furthermore, Burkholderia DSF was reported to upregulate the genes involved in iron acquisition in F. novicida, which increased siderophore production [72].

Subsequently, we compared the metabolites in the broth of B. subtilis trpC2 and trpC2 yxeJ (Figure 2). Among the 3150 detected peaks, the intensities of 40 peaks were significantly higher in the broth of B. subtilis trpC2 compared to that of trpC2 yxeJ (Table S4). The structures of 32 significantly detected compounds in B. subtilis trpC2 broth were estimated by MS/MS analysis (Table 2 and Table S5). Diverse peptides were detected in B. subtilis trpC2 broth. Nicotinamide reportedly enhances growth of both Gram-negative and Gram-positive bacteria, such as M. avium, Propionibacterium acnes, S. aureus, and B. macerans [73–76]. Indole-3-carboxaldehyde was shown to efficiently inhibit biofilm formation by Vibrio cholerae O1 [77]. The utilization of urocanic acid by Pseudomonas and Aeromonas strains has been reported [78,79]. Nopaline is a carbon and nitrogen source.
metabolized by *Agrobacterium*. 6-Paradol was reported to have significant anti-adhesive activity against *S. aureus* [80].

### Table 2. The constituents in *B. subtilis trpC2* broth detected by LC-MS/MS analysis.

| No. | Formula     | Exact Mass | Name                                               | Database | Database ID   |
|-----|-------------|------------|----------------------------------------------------|----------|---------------|
| 1938| C₁₂H₂₂O₈N  | 309.142    | 4-O-beta-D-Glucopyranosylagomine                   | UC2      | C00049954     |
| 1980| C₁₅H₂₃O₂₂S₂ | 445.233    |                                                    | EX-HR2   |               |
| 453 | C₁₄H₃₀O₆N₆  | 402.223    |                                                    | EX-HR2   |               |
| 2242| C₁₂H₃₁O₄N₃  | 341.231    | Diprotin A                                         | UC2      | C00018579     |
| 1607| C₂₅H₄₀O₇    | 452.277    | Briarellin P                                       | UC2      | C00044586     |
| 799 | C₁₀H₂₉O₂N₂S₂ | 248.119    | Valyl-Methionine                                   | UC2      | HMDB0029133   |
| 1607| C₁₀H₂₉O₂N₂S₂ | 248.119    | Methionyl-Valine                                   | UC2      | HMDB0028986   |
| 510 | C₁₁H₂₂O₄N₄  | 274.164    | Glutaminyllysine                                   | UC2      | HMDB0028802   |
| 2242| C₁₁H₂₂O₄N₄  | 274.164    | Lysyl-Gamma-glutamate                              | UC2      | HMDB0028965   |
| 960 | C₂₁H₄₀O₃N₃P₃ | 443.238    |                                                    | EX-HR2   |               |
| 2575| C₆H₁₃N₂P₂    | 213.058    |                                                    | EX-HR2   |               |
| 2345| C₁₉H₂₅N₃O₅S | 395.188    | V1M1F1                                             | Pep1000  |               |
| 2536| C₂₅H₃₆O₅N₄  | 520.269    | Lotusine F                                         | UC2      | C00027221     |
| 2536| C₂₅H₃₆O₅N₄  | 520.269    | Nummularine S                                      | UC2      | C00029150     |
| 2237| C₃₃H₄₄O₃₁   | 616.288    | Neoazedarachin A                                   | UC2      | C00039833     |
| 2633| C₂₃H₅₃O₁₂N₁P₂ | 599.320    |                                                    | EX-HR2   |               |
| 2673| C₄₆H₆₇O₂₃N¹₃P₁S₁ | 854.491  |                                                    | EX-HR2   |               |
| 1271| C₂₇H₄₄O₇    | 512.299    | Butyrolactol B                                     | UC2      | C00016754     |
| 1271| C₂₇H₄₄O₇    | 512.299    | Integristerone B                                   | UC2      | C00048431     |
| 1271| C₂₇H₄₄O₇    | 512.299    | Platenolide B mycarose                             | UC2      | C00018288     |
| 162 | C₄H₄ON₂     | 122.048    | Nicotinamide                                       | UC2      | C00000209     |
| 162 | C₄H₄ON₂     | 122.048    | 2-Acetylpyrazine                                   | UC2      | HMDB0031861   |
| 1710| C₁₅H₂₄O₄N₄  | 324.180    |                                                    | EX-HR2   |               |
| 211 | C₄H₄O₃N     | 143.058    | SQ 26517                                          | UC2      | C00018434     |
| 211 | C₄H₄O₃N     | 143.058    | Trimethadione                                      | UC2      | HMDB0014491   |
| 211 | C₄H₄O₃N     | 143.058    | 6-Oxopiperidine-2-carboxylic acid                  | UC2      | HMDB0061705   |
| 211 | C₄H₄O₃N     | 143.058    | 5-ethyl-5-methyl-2,4-oxazolidinedione              | UC2      | HMDB0061082   |
| 211 | C₄H₄O₃N     | 143.058    | Vinylacetylglycine                                 | UC2      | HMDB0000894   |
| 211 | C₄H₄O₃N     | 143.058    | Methyl pyroglutamate                               | UC2      | C00051578     |
| 1258| C₂₂H₆₆N₂₃P₃S₆ | 612.303    |                                                    | EX-HR2   |               |
| 994 | C₂₀H₃₃N₅O₈  | 471.233    | G2[I;I]I[E1P1, G1A1V1E1P1, G1A1[I;I]I[D1P1, G1T2P2, A2V1D1P1, A1S1T1P2, V1E1Q1P1, [L;I]I]I]1Q1P1, [L;I]I]2E1N1P1 | Pep1000  |               |
| 655 | C₁₆H₂₇N₃O₆  | 385.196    | G3V1P1, G1A3P1, G1V1N1P1, A2Q1P1                    | Pep1000  |               |
| No. | Formula | Exact Mass | Name | Database | Database ID |
|-----|---------|------------|------|----------|-------------|
| 1034 | C₁₀H₁₆O₃N₂ | 212.116 | Butabarbital | UC2 | HMDB0014382 |
|     | C₁₀H₁₆O₃N₂ | 212.116 | L-prolyl-L-proline | UC2 | HMDB0011180 |
|     | C₁₀H₁₆O₃N₂ | 212.116 | Butethal | UC2 | HMDB0015442 |
| 457  | C₃₂H₄₄O₅N₂S₁ | 572.328 | EX-HR2 |
| 2755 | C₄H₇O | 145.053 | Indole-3-carboxaldehyde | UC2 | C00000112 |
|     | C₄H₇O | 145.053 | 2-Quinolone | UC2 | C00044432 |
| 2680 | C₆₇H₁₉₈O₉N₂S₅ | 1196.681 | EX-HR2 |
| 115  | C₉H₆O₂N₂ | 138.043 | 4-Methoxylonchocarpin | UC2 | HMDB0031338 |
|     | C₉H₆O₂N₂ | 138.043 | 2-Aminonicotinic acid | UC2 | HMDB0061680 |
|     | C₉H₆O₂N₂ | 138.043 | Urocanic acid | UC2 | HMDB0062562 |
|     | C₆H₆O₂N₂ | 138.043 | Nicotinamide N-oxide | UC2 | HMDB0002730 |
| 2949 | C₁₁H₁₂O | 183.162 | Tecostanin | UC2 | C00001984 |
|     | C₁₁H₁₂O | 183.162 | Incarvilline | UC2 | C00050294 |
| 1600 | C₂₀H₂₅O₄N₂S₅ | 583.370 | EX-HR2 |
| 1727 | C₁₇H₂₀O₄N₄ | 304.138 | Nopaline | UC2 | C00001548 |
| 526  | C₃₁H₅₆O₁₄N₁₀P₂ | 734.345 | EX-HR2 |
| 3061 | C₁₇H₂₅O₃ | 278.188 | 1-Acetoxy-3,15-epoxygymnomitrane | UC2 | C00021889 |
|     | C₁₇H₂₅O₃ | 278.188 | Litsealactone B | UC2 | C00044889 |
|     | C₁₇H₂₅O₃ | 278.188 | 9beta-Acetoxy-10(14)-aromadendren-4beta-ol | UC2 | C00021235 |
|     | C₁₇H₂₅O₃ | 278.188 | Furoscrobiculin C | UC2 | C00021531 |
|     | C₁₇H₂₅O₃ | 278.188 | [S-[R *,S *-(E)]]-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene-4,5-diol 4-acetate | UC2 | C00049252 |
|     | C₁₇H₂₅O₃ | 278.188 | Panaxytriol | UC2 | C00030923 |
|     | C₁₇H₂₅O₃ | 278.188 | Panaxacol | UC2 | HMDB0039251 |
|     | C₁₇H₂₅O₃ | 278.188 | Parahigginol C | UC2 | C00009500 |
|     | C₁₇H₂₅O₃ | 278.188 | 8alpha-Hydroxygymnomitrinylacetate | UC2 | C00021248 |
|     | C₁₇H₂₅O₃ | 278.188 | Lincomolide B | UC2 | C00047968 |
|     | C₁₇H₂₅O₃ | 278.188 | 4alpha-Hydroxystrobilurin | UC2 | C00021894 |
|     | C₁₇H₂₅O₃ | 278.188 | 4-{(4E)-3-hydroxydec-4-en-1-yl}-2-methoxyphenol | UC2 | HMDB0137260 |
|     | C₁₇H₂₅O₃ | 278.188 | Ro 09-1544 | UC2 | C00017230 |
In this study, we demonstrated that constituents in membrane vesicles significantly promoted the growth rate of \textit{C. thermocellum}. Additionally, the MV constituents with growth stimulation were described by LC-MS/MS analysis. These findings suggest that the constituents in membrane vesicles could promote \textit{C. thermocellum} growth, leading to improved efficiency of cellulosic biomass utilization.

**Supplementary Materials**: The following are available online at https://www.mdpi.com/2076-2607/9/3/593/s1, Figure S1: MVs from \textit{C. thermocellum} and \textit{B. subtilis}. Figure S2: Effect of myo-inositol on \textit{C. thermocellum} growth. Figure S3: Effect of pyrrolo-quinoline quinone on \textit{C. thermocellum} growth. Table S1: Genotypes of \textit{B. subtilis} genome deletion mutants. Table S2: \textit{B. subtilis} single gene deletion mutants used in this study. Table S3: Intensities of detected peaks in the MV fraction of \textit{C. thermocellum} by LC-MS/MS. Table S4: Intensities of the detected peaks in cell-free \textit{B. subtilis trpC2} broth by LC-MS/MS. Table S5: Structures of constituents detected by LC-MS/MS in this study.

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