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RESEARCH PAPER

Bioconversion of Plant Hydrolysate Biomass into Biofuels Using an Engineered *Bacillus subtilis* and *Escherichia coli* Mixed-whole Cell Biotransformation

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Abstract Bioconversion of organic biomass such as plant hydrolysate and organic waste into valuable biochemicals is very challenging. In this study, carbohydrate-rich watermelon rinds and protein-rich okara (soybean waste) were converted into biofuels of ethanol, isobutanol, and methylbutanols using an engineered *Escherichia coli* and *Bacillus subtilis* mixed-whole cell biotransformation. The engineered *E. coli* expressed the genes *alsS*, *kivD*, *ilvC*, *ilvD*, and *yqhD*, and the engineered *B. subtilis* expressed the genes *leuDH*, *kivD*, and *yqhD*. The growth inhibition of the *B. subtilis* strain, which was reduced by 50% with addition of 1 mM furfural, was restored by the addition of 1 g/mL of activated carbon. The ratio of the *E. coil* and *B. subtilis* was optimized depending on carbohydrate and protein composition of the hydrolysate. When the carbohydrate levels were high, a 4:1 ratio of engineered *E. coli* to *B. subtilis* led to the highest overall biofuel (1.1 g/L) and isobutanol (80%) production. Viability analysis of the engineered *E. coli* to *B. subtilis* strains showed that the *E. coli* strain had higher activity at the beginning of the biotransformation period, while the *B. subtilis* strain exhibited higher activity in the later stages. The results of the present study provide important information for future biochemical production research, particularly regarding the diversification of organic waste resources.

Keywords: plant hydrolysate biomass, organic waste conversion, *Bacillus subtilis*– *Escherichia coli* mixed biotransformation, biofuels

1. Introduction

The demand for sustainable energy resources has steadily increased due to concerns about environmental pollution and the exhaustion of fossil fuels. As such, research on the chemical and biological production of industrially useful energy sources using various feedstocks, including organic waste, has attracted significant attention. Organic waste, which is typically composed of various nutrients, is a valuable resource [1-3], but separation or pretreatment with organic solvents to produce complex organics is required due to variation in its carbohydrate, protein, and fat composition [4-6]. Therefore, it is important to convert these complex organics into useful biochemicals using one-pot bioprocesses [7].

In terms of the conversion of carbohydrates, which make up the highest proportion of the composition of organic matter, many studies have reported the use of *Escherichia coli* with glucose as the carbon source to produce, for example, C2–C5 biofuels. According to recent results reported by Yang's group, *E. coli* cells expressing enzymes in the Ehrlich pathway are able to produce up to 6 g/L of isobutanol from 20 g/L of glucose in 48 h [8-10]. The engineered *E. coli* strain was used as a bioconversion host, expressing five genes: *alsS* (acetolactate synthase), *kivD* (2-ketoisovalerate decarboxylase), *ilvC* (ketol-acid reducto-

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isomerase), *ilvD* (dihydroxy acid dehydratase), and *yqhD* (alcohol dehydrogenase). In this reaction, AlsS converts pyruvate, which is one of the main intermediates in glucose metabolism, into acetolactate, followed by IlvCD reactions generating 2-ketoisovalerate. Finally, the 2-ketoisovalerate is converted into isobutanol through KivD and YqhD enzymes. As glucose is one of the most useful carbon sources for *E. coli* host strains and solubilizes well in media without any treatment, biofuel production using *E. coli* hosts has been very successfully achieved to date.

Contrary to carbohydrate, the conversion of protein-rich organic waste, in particular the direct conversion of amino acids using microbial bioconversion processes, remains a challenge [11]. One of the limitations of protein-based biorefinery is that it requires protease and hydrolysis pretreatment, or protease-producing host cells should be utilized. Recently, our group reported the bioconversion of protein-rich spent coffee grounds and okara (soybean waste) into biofuels [12]. In this research, engineered *Bacillus subtilis* was utilized as it could produce endogenous protease and permease enzymes, otherwise protease-dependent hydrolysis process is required [12]. The *Bacillus* system has the advantages of consolidated bioprocesses as it does not require protein hydrolysis or pretreatment process [11,13]. In a previous report, a *B. subtilis* expressing *leuDH* (leucine dehydrogenase), *kivD*, and *yqhD* genes directly converted branched-chain amino acids (BCAAs) into the biofuels isobutanol, 2-methylbutanol, and 3-methylbutanol, respectively [11,14]. In addition, the global regulator CodY, which is involved in BCAA metabolism, and BkdB, which is involved in 2-keto acid degradation, were both removed to avoid transcriptional regulation by BCAAs [7].

As mentioned above, the biotransformation of various nutrients has progressed considerably, but it remains necessary to develop a technology that integrates and converts them simultaneously in order to decrease operational complexity and costs. One possible approach is to develop a single versatile host strain that can convert carbohydrates and proteins at the same time [15]. However, this strategy faces a number of catabolic hurdles, such as catabolite repression and a high metabolic burden [16,17]. Another solution is to develop two separate host strains and overcome these challenges via mixed-whole cell biotransformation [18-20], but combining two different cells in a single biotransformation process is not easy due to differences in cell viability and biotransformation activity. In addition, maintaining a consistent composition of organic waste during microbial conversion is generally regarded to be important because any imbalance could affect the growth of the host cells, reducing the conversion of organic waste. Thus, optimizing a mixed-whole cell biotransformation system for the compositional ratio dictated by the organic waste being converted in order to produce the desired biofuels and homogeneous biochemicals is a significant challenge that needs to be overcome.

In the present study, we attempt to overcome the limitations of organic waste biomass utilization caused by the inhibitory activity of byproducts and differences in the compositional ratio by engineering *B. subtilis* and *E. coli* strains. First, acetic acid and furfural aldehyde, which are produced as byproducts in the biotransformation process, were removed by adsorption [9,21]. The removal of these inhibitory byproducts positively affected cell mass and biofuel production. We then utilized watermelon rinds (a carbohydrate source) and dried okara (a protein source) as substrates among the examined several plant hydrolysates for the *E. coli* and *B. subtilis* mixed-reaction system. It was confirmed that the higher production of bioalcohol from different compositional ratios of the food waste could be achieved by optimizing the cell-mass ratio of the engineered *E. coli* and *B. subtilis* cells. The resulting process provides important insights into the utilization of various forms of complex organic biomass for further research. Furthermore recycling strategies of generated by-products such as crude glycerol and free fatty acids were suggested to achieve total conversion of plant hydrolysates without carbon losses.

2. Materials and Methods

2.1. Chemicals, reagents, and raw materials

Yeast extract (Technical, Bacto^{TM}) was obtained from BD Bioscience Korea (Seoul, Korea). All chemicals and reagents used in the experiments were purchased from Sigma-Aldrich (Suwon, Korea). Watermelon rinds and okara were kindly supplied by a local market (Ajou University, Suwon, Korea).

2.2. Bacterial strains, media, and culture conditions

Minimal medium was prepared by mixing 1x trace element (containing 10 mg/L $MnCl_2{\cdot}4H_2O$, 17 mg/L $ZnCl_2$, 3.3 mg/L $CuCl_2{\cdot}2H_2O$, 6 mg/L $CoCl_2$ $6{\cdot}H_2O$, and 6 mg/L $Na_2MoO_2{\cdot}2H_2O$) with M9 salts (containing 6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH4Cl, and 0.5 g/L NaCl). The final concentration of glucose as a carbon source and BSA as a protein source is 1 g/L, respectively. Engineered *B. subtilis* was inoculated into 40 g/L yeast extract medium and engineered *E. coli* was inoculated into 25 g/L Luria-Bertani broth. *B. subtilis* strain was described in the previous report [7]. A yeast extract medium contains 40 g/L of Bacto Yeast Extract mixed with M9 salt. For *B. subtilis* strain culture, 100 μg/mL spectinomycin, 5 μg/mL chloramphenicol, and 1 μg/mL erythromycin were used. *E. coli* DSM01 strain (K12 MG1655 *ΔldhA*::FRT, *ΔadhE*::FRT, *ΔfrdA*::FRT,

Δpta::FRT) expressing harboring both of pCDFDuet-1::*alsS*, *kivD* and pET23a::*ilvC*, *ilvD*, *yqhD* were prepared reported previously [8], while a *B. subtilis 168* (KCTC No. 1326)Δ*codY*Δ*bkdB* strain harboring pHCMC05::*lueDH*, *kivD*, *yqhD* was prepared as previously described [7]. Plasmids expressing *alsS*, *kivD*, *ilvCD*, *yqhD* were controlled T7 promoter, while *lueDH*, *kivD*, *yqhD*, *yqhD* genes were controlled under a P_{spac} promoter in the pHCMC05 backbone.

2.3. Gene expression for the organic food waste conversion process

To prepare the *E. coli* cells, 2 mL of seed culture in LB was incubated at 37°C for 12 h at 250 rpm shaking incubator. Following this, 0.5 mL of the seed culture was inoculated into 0.1 mL of LB medium (250 mL flask), and incubated at 37°C of shaking incubator (250 rpm). And then the cells were induced with 0.1 mM isopropyl-*β*-D-thiogalactoside until the OD_{600} reached around 0.8, and further incubated for another 24 h at 30°C.

To prepare the *B. subtilis* cell mass, 2 mL of seed culture in a 40 g/L yeast extract medium was incubated at 37°C for 12 h at 250 rpm shaking incubator, after which 0.5 mL of the seed culture was inoculated into 25 mL of same medium (250 mL flask). The cells were grown at 37°C until the OD_{600} 0.8, and then the genes encoding LeuDH, KivD, and YqhD enzymes in the plasmids were induced with 3 mM IPTG simultaneously [7].

2.4. Pretreatment process for the watermelon rind and okara waste

The watermelon rinds and okara were prepared by drying them in the oven, followed by homogenization. After 1 h of heating and filtering, the aqueous phase was isolated with a 50 mM sodium citrate buffer (pH 5, 20% w/w). Following this, 100 g each of the dried watermelon rinds and okara dry powder was autoclaved (121°C, 30 min), followed by purification by filtration. The filtered liquid phase from the watermelon rinds and okara was prepared for fermentation using the engineered *E. coli* and *B. subtilis* mixed-whole cell biotransformation at 37°C.

2.5. Bioconversion of the watermelon rind and okara waste into biofuels

For mixed-whole cell biotransformation fermentation, engineered *B. subtilis* and *E. coli* derived from each 250 mL flask are incubated overnight separately, then mixed with an appropriate proportion (1:1, 1:4, 1:9, *etc*.) after washing to ferment in the waste biomass hydrolysates. The cap-sealed flasks were fermented at 37°C, 200 rpm for 5 days for biofuels production and the biofuels production was monitored daily.

2.6. Separation and quantification of biofuels of ethanol, isobutanol, and methylbutanols

Each 1 mL sample was collected hourly for quantitative analysis of generated biofuels. For sample pretreatment, the supernatant of the sample was separated by centrifugation (13,500 rpm, 4°C, 10 min). And the separated supernatants were filtered by syringe and prepared for gas chromatography (GC) analysis. Biofuels of ethanol, isobutanol, and methylbutanols were quantified using a GC analysis. The instrumental information of GC analyzer; a flame ionization detector (225°C), split ratio: 1:5 of split injection, and capillary column DB-FFAP, inside diameter 30 m × 0.25 m, 0.25 μm thickness (Younglin, Seoul, Korea) [22]. The initial oven temperature was set at 50°C and raised to 200°C at a temperature gradient of 15°C/min.

3. Results and Discussion

3.1. Mixed-whole cell biotransformation system of engineered *E. coli* and *B. subtilis* for protein and carbohydrate conversion

The two bacterial strains were able to convert proteins and carbohydrates into C2–C5 biofuels. The *E. coli* strain expressing *als*, *kivD*, *ilvC*, *ilvD*, and *yqhD* converted carbohydrates [8,10], while the *B. subtilis* strain, in which CodY and BkdB were deleted and *leuDH*, *kivD*, and *yqhD* overexpressed, converted the proteins [7]. Using these two strains, a mixed-whole cell biotransformation system that could simultaneously convert water-soluble carbohydrates

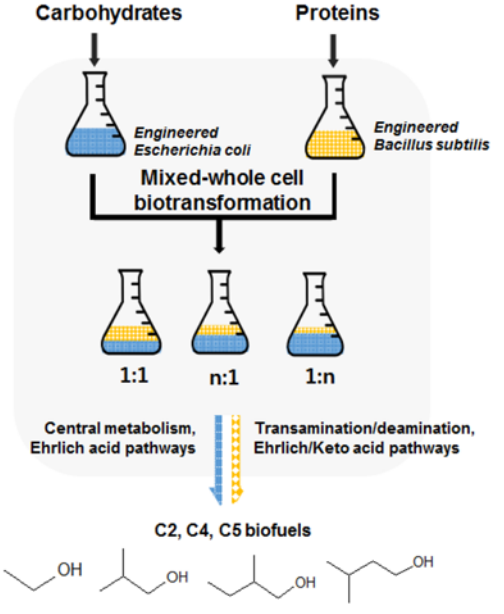

Fig. 1. Mixed-whole cell biotransformation system for converting carbohydrate and protein waste into C2–C5 biofuels using various ratios of engineered *Escherichia coli* and *Bacillus subtilis*.

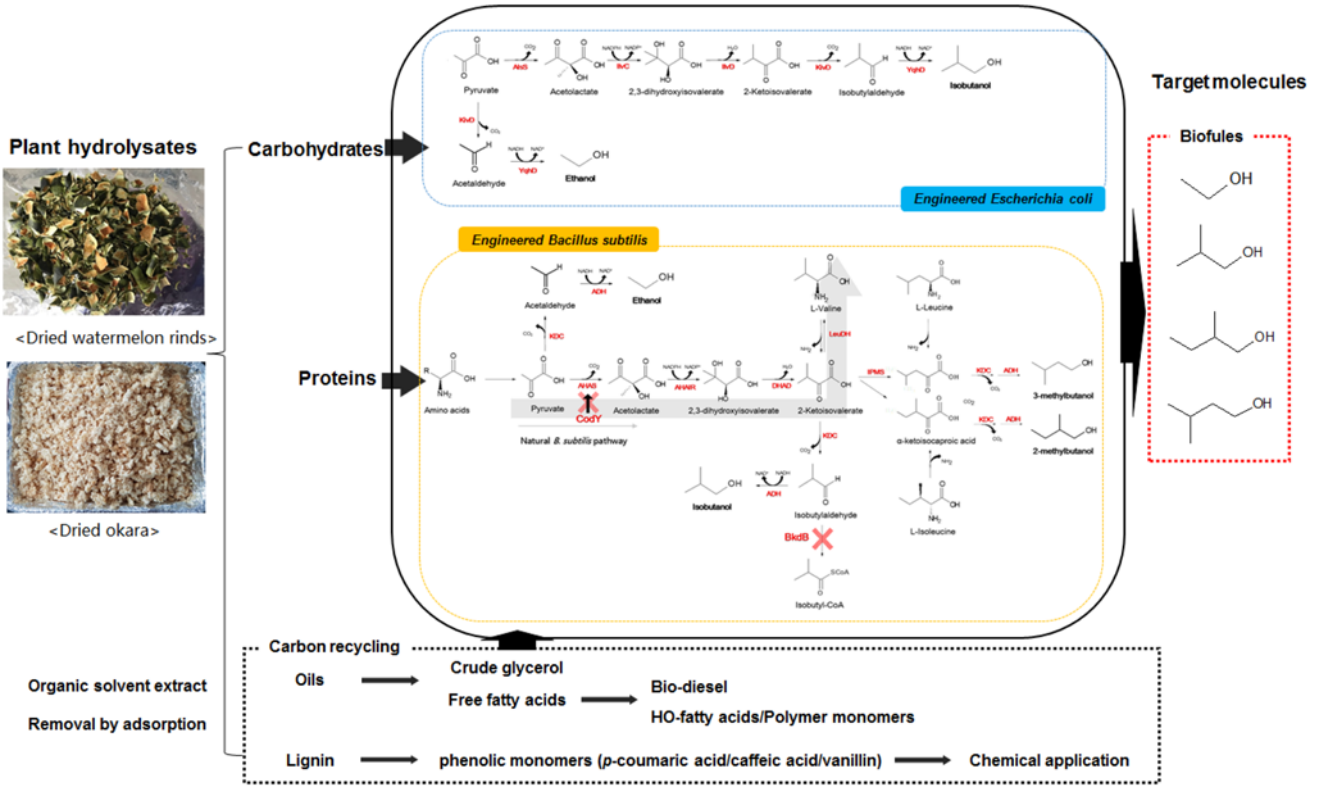

Fig. 2. Mixed-whole cell biotransformation system for the production of C ≥ 2 bioalcohols (dotted box at the right) from carbohydrate and protein biomass using engineered *Escherichia coli* and *Bacillus subtilis* strains. *E. coli* expressed *alsS*, *kivD*, *ilvC*, *ilvD*, and *yqhD*, while *B. subtilis* expressed *leuDH*, *livD*, and *yqhD*, with CodY and BkdB removed. *E. coli* and *B. subtilis* biosynthetic pathways for BCAAs via intracellular *ilv-leu* operons, including AHAS and IlvCDE. And recycling strategies of the generated lignin monomers and oil by-product of crude glycerol and free fatty acids during hydrolysate extraction from plant hydrolysate biomass for further development in consolidated bioprocess were suggested (dotted box at the bottom).

and proteins was established, with an end product of C2–C5 biofuels of ethanol, isobutanol, 2-methylbutanol, and 3-methylbutanol (Fig. 1). In carbohydrate metabolism, the Ehrlich pathway produces isobutanol by converting the pyruvate produced during glycolysis, while the Ehrlich pathway converts proteins by producing 2-keto acid via the dehydrogenation of BCAAs (Fig. 2).

3.2. Removal of the bioconversion byproducts acetic acid and furfural via adsorption

One of the most bottle neck in plant-based biomass conversion is the inhibitory effect of the plant components such as furfural, lignin monomers of phenolic compounds, and other fermentative chemicals. When selecting the organic biomass for bioconversion, we have screened several plant-based biomass such as barley straw and waste coffee ground as carbohydrate and protein-waste representatives, respectively [23]. However, the inhibitory compositions such as phenolic lignin and furfural in their hydrolysate greatly affected the cell growth and bio-alcohol production in spite of their abundant nutritional resources. In such cases it seems rather more efficient in the production of fatty acids by converting lignin or by fermentation [24,25]. Therefore removal of inhibitory components and bio-alcohol production studies were carried out using watermelon rind and okara as substrates with relatively low lignin content in this study.

First, byproducts such as acetic acid and furfural produced during the conversion of carbohydrates can lower biofuel production due to cell-growth inhibition. The inhibition of cell growth can be prevented using enzymes that regulate the pathways that remove these byproducts from biotransformed strains or that regulate redox potentials. However, when compositionally complex organic waste is used as a source, various byproducts are generated and would affect several metabolic pathways which cannot be overcome by metabolic engineering. As one of possible alternatives in this study, an activated carbon was added for an adsorptive inhibitor removal during the bioconversion process. Furfural is produced by ethanogenic *E. coli*, and it was believed that it may inhibit the growth of *B. subtilis*. In addition, protein conversion by the *B. subtilis* strain may inhibit the growth of the *E. coli* strain due to a change in the pH and the higher cytotoxicity because of the secondary production of

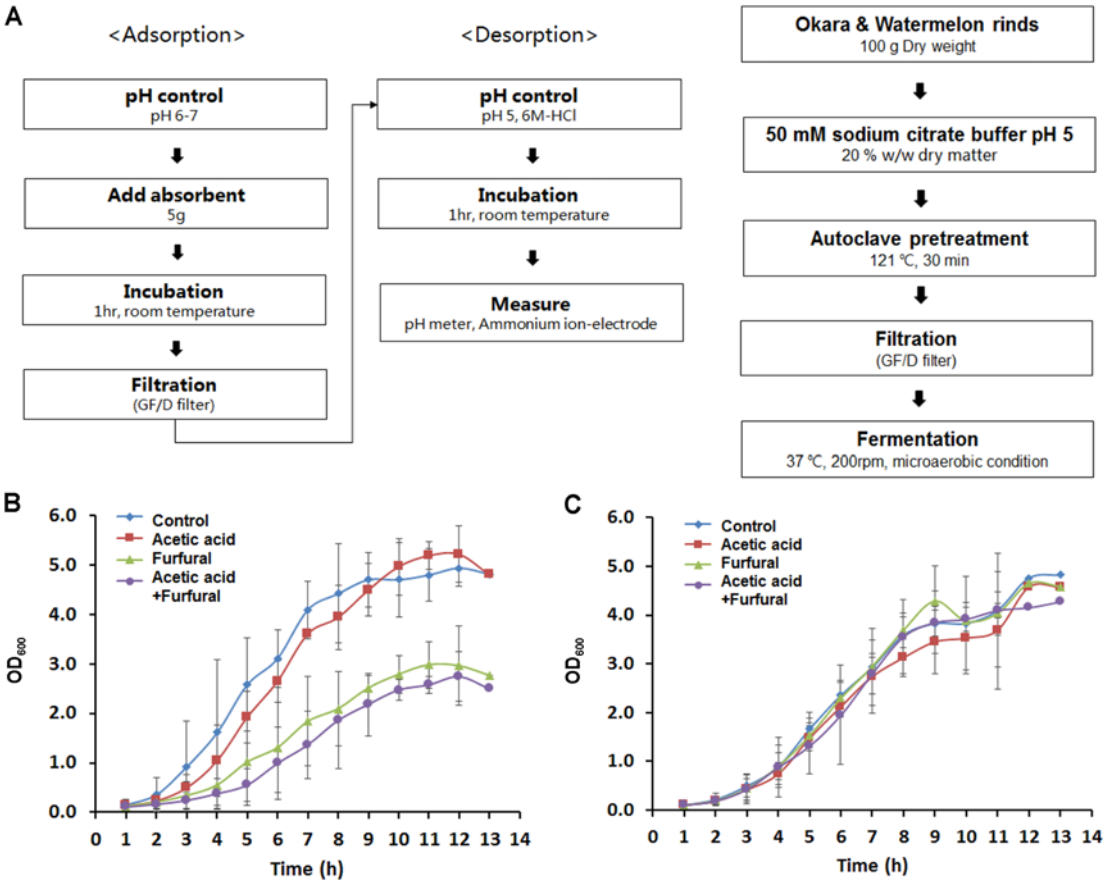

Fig. 3. Preparation of adsorptive removal of inhibitory chemicals and plant hydrolysate for mixed-whole cell biotransformation. (A) Adsorptive removal of acetic acid and furfural using activated carbon and quantification of the byproducts removed using desorption. Pretreatment process for the bioconversion of watermelon rinds and okara protein biomass in mixed-whole cell biotransformation fermentation. (B) Cell-growth inhibition of engineered *Bacillus subtilis* strain by the byproducts of acetic acid and furfural. (C) Cell-growth recovery of engineered *B. subtilis* strain following the adsorptive removal of furfural using activated carbon.

ammonia.

The effect of acetic acid and furfural on cell growth in the *B. subtilis* strain and its subsequent recovery after these byproducts were removed via adsorption using activated carbon were analyzed (Fig. 3A). In the *B. subtilis* strain, no growth inhibition by acetic acid was observed, but cell growth was reduced by 50% by addition of 1 mM furfural (Fig. 3B). In order to remove the furfural by adsorption, the cells were simultaneously cultured with 0.1 g/mL of activated carbon; as a result, it was confirmed that the inhibition of cell growth by furfural was completely recovered (Fig. 3C).

3.3. Mixed-whole cell biotransformation of *B. subtilis* and *E. coli* cells at different ratios in a yeast extract medium

In order to verify the feasibility of the mixed-whole cell biotransformation system for the engineered *E. coli* and *B. subtilis* strains, bioconversion using the YEM supplemented with M9 salts was investigated first. The biofuel production of the *E. coli* and *B. subtilis* strains in YEM at mass ratios of 1:1, 1:1.5, and 1.5:1 (*E. coli*: *B. subtilis*) cells was examined. The engineered *E. coli* strain produced a significantly higher biofuel production of 6 g/L with the 40 g/L YEM with isobutanol and methylbutanols as main products (Fig. 4A). In the mixed-whole cell biotransformation system, the total biofuel production decreased to 2 g/L as the ratio of the *B. subtilis* strain increased (Fig. 4B). This is because the protein biomass conversion efficiency of *B. subtilis* was lower than that of *E. coli* of 5 g/L, indicating that the conversion activity of *E. coli* makes a higher contribution to the system. Therefore, it is necessary to optimize the ratio of *E. coli* and *B. subtilis* cell mass according to the ratio of carbohydrates to protein in the organic waste source.

3.4. Conversion of plant-derived food hydrolysate by *E. coli* and *B. subtilis* cells

The bioconversion of organic plant hydrolysates of food waste into biofuels was then conducted based on the results obtained from the defined YEM-based bioconversion experiments. Carbohydrate-rich watermelon rinds and

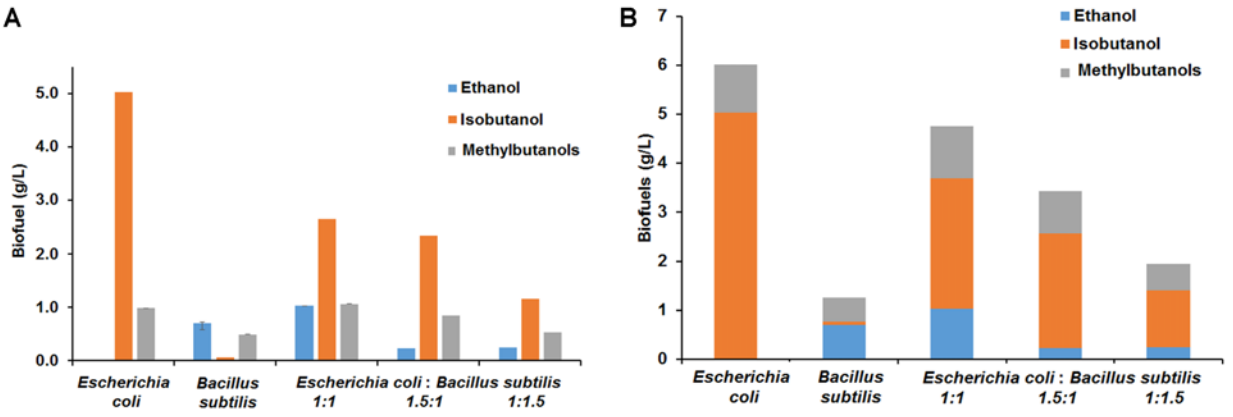

Fig. 4. (A) Conversion of 40 g/L of yeast extract into ethanol, isobutanol, and methylbutanols using a mixed-whole cell biotransformation of the engineered *Escherichia coli* and *Bacillus subtilis* strains with different cell ratios. (B) Total biofuel production and its composition by engineered *E. coli* and *B. subtilis* cells and mixed-whole cell reaction.

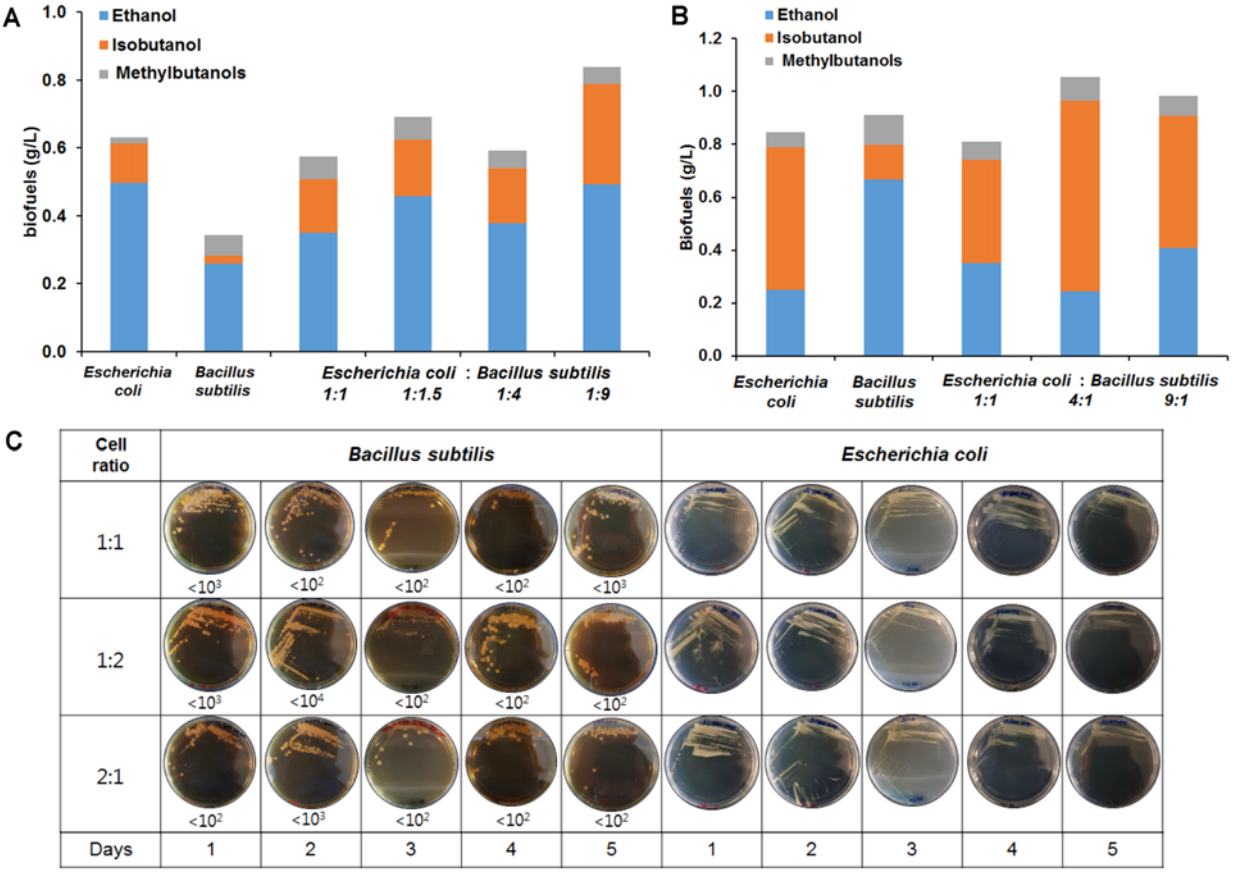

Fig. 5. Bioconversion of the watermelon rinds and okara biomass was conducted with (A) 0.05 g/L of glucose and 0.2 g/L of protein and (B) 0.5 g/L of glucose and 0.05 g/L protein in an mixed-whole cell biotransformation with different engineered *Escherichia coli* and *Bacillus subtilis* cell ratios. (C) Cell viability monitoring of the engineered *E. coli* and *B. subtilis* strains via colony formation on separate agar plates during the food-waste bioconversion process. The numbers under the *B. subtilis* plates indicate the rough approximation of generated colony formation numbers. The cell ratio; *E. coil* and *B. subtilis*.

protein-rich okara were selected as representative organic food waste among the examined plant hydrolysates. The individual carbohydrate- and protein-rich solutions were autoclaved and filtered for the preparation of the fermentation broths (Fig. 3A). After mixing the food hydrolysate broths with M9 salts in 50 mL scale, different ratios of the two strains of bacterial cell were added to the incubation broths.

For protein-rich broth, which contained 0.05 g/L of glucose

and 0.2 g/L of protein in the mixture of watermelon rinds and okara hydrolysate, the *E. coli* and *B. subtilis* cells produced approximately 0.6 g/L and 0.3 g/L of biofuels, respectively (Fig. 5A). Because the okara hydrolysate had high protein composition, biofuel production was monitored with the increase of the proportion of *B. subtilis* cells. As the *E. coli* to *B. subtilis* cell mass ratio changed from 1:1 to 1:1.5, 1:4, and 1:9, the biofuel production increased gradually and the highest titer of 0.8 g/L biofuels was achieved when the cell ratio of 1:9, which was 40% higher than that achieved for *E. coli* alone (Fig. 5A).

For carbohydrate-rich broth, which contained 0.5 g/L glucose and 0.05 g/L protein in the mixture of watermelon rinds and okara hydrolysate, bioconversion activity was measured by increasing the *E. coli* cell mass ratio on the contrary. In this case, the use of *B. subtilis* alone led to higher biofuel yields than the use of *E. coli* alone. This might be because the higher glucose levels could lead the ethanol fermentation by the engineered *B. subtilis* strain to a greater extent than the efficiency of the conversion to isobutanol by the *E. coli*. Biofuel production was highest (1.1 g/L) when the *E. coli* to *B. subtilis* ratio was 4:1, with the highest proportion of isobutanol at about 80% (Fig. 5B). Interestingly, biofuel productivity did not steadily increase with a higher proportion of *E. coli* cells with a glucose-rich substrate. Rather, the increase in ethanol fermentation from glucose lowered isobutanol production relatively and the total biofuel production fell back to the similar level when engineered *E. coli* was used alone.

3.5. *E. coli* and *B. subtilis* cell viability during bioconversion process

In mixed-whole cell biotransformation systems, especially when using gram-negative and gram-positive strains, it is important to ensure that the activity of each cell is maintained independently during the bioconversion process. In order to evaluate the physiological activity of the *E. coli* and *B. subtilis* strains in the mixed-whole cell biotransformation system when the strain ratios were 1:1, 1:2, and 2:1, cell viability was observed by colony formation during the conversion process.

When the cell ratio was 1:1, the colonies of the engineered *B. subtilis* cells tended to continuously decrease up to four days of incubation and then increased after five days in the mixed-whole cell biotransformation, whereas *E. coli* maintained its cell mass for four days and then decreased after five days (Fig. 5C). A brief decrease in *B. subtilis* cells during biotransformation and gradual decrease in *E. coli* cells after four days of bioconversion were also observed when the mixing ratio was 1:2 and 2:1 as well. These findings suggested that the conversion of biofuels was dominated by the *E. coli* strain at the beginning of the biotransformation process, while the *B. subtilis* strain dominated in the latter stage of biotransformation. The *E. coli* and *B. subtilis* mixed-whole cell biotransformation system seemed to be effective when the glucose composition ratio was high because *E. coli*, which has high bioconversion activity, was able to efficiently convert the carbohydrates at the beginning of the plant hydrolysate biomass conversion process.

4. Conclusion

In this study, plant-derived food hydrolysate was converted into biofuels using an engineered *E. coli* and *B. subtilis* mixed-whole cell biotransformation. Dry powders produced from carbohydrate-rich watermelon rinds and protein-rich okara were used as the substrates, and the engineered *E. coli* and *B. subtilis* strains with different ratios were utilized for ethanol, isobutanol, and methylbutanols production.

First, activated carbon was used to restore *B. subtilis* growth levels, which are negatively affected by the acetic acid and furfural produced by ethanogenic *E. coli* [26]. Next, the optimal ratio of the *E. coli* and *B. subtilis* strains was optimized according to the carbohydrate and protein composition of the watermelon rinds and okara. Cell viability analysis of the *B. subtilis* and *E. coli* strains found that the *E. coli* strain had higher activity at the beginning of the biotransformation process, while the *B. subtilis* strain became dominant later. Mixed-whole cell biotransformation system represent an efficient bioprocess for the treatment of various forms of organic waste that differ in their nutrient composition [18,20].

However, in order to ensure high productivity and the desired final product composition, an optimization process is necessary. For example, the optimal ratio of the cell masses and maximization of the cell activity originating from interactions between the cells should be achieved. Furthermore, it may be very useful to recycle the generated oil by-product during hydrolysate extraction from plant biomass (Fig. 2, dotted box at the bottom). The wasted oil could be further hydrolyzed into crude glycerol, which could be recycled for microbial carbon sources, and free fatty acids such as oleic and ricinoleic acid, which could be further applied for hydroxyfatty acid or fatty acid methylester forms of bio-diesel [23,25-27]. Besides several valuable chemicals such as caffeic acid, *p*-coumaric acid, ferulic acids as major antioxidant phenolics in watermelon rinds, which were removed or not utilized in this study, and could be further recycled [28]. These results will provide important information for future biochemical production efforts on the diversification of organic waste resources.

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The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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