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ORIGINAL ARTICLE

Synbiotic Fermentation of *Undaria pinnatifida* and *Lactobacillus brevis* to Produce Prebiotics and Probiotics

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Abstract
It has been optimized thermal acid hydrolytic pretreatment and enzymatic saccharification (*E*s) in flask culture of *Undaria pinnatifida* seaweed, which is a prebiotic. The optimal hydrolytic conditions were a slurry content of 8% (w/v), 180 mM H_2SO_4, and 121°C for 30 min. *E*s using Celluclast 1.5 L at 8 U/mL produced 2.7 g/L glucose with an efficiency of 96.2%. The concentration of fucose (a prebiotic) was 0.48 g/L after pretreatment and saccharification. The fucose concentration decreased slightly during fermentation. Monosodium glutamate (MSG) (3%, w/v) and pyridoxal 5′-phosphate (PLP) (30 μM) were added to enhance gamma-aminobutyric acid (GABA) production. To further improve the consumption of mixed monosaccharides, adaptation of *Lactobacillus brevis* KCL010 to high concentrations of mannitol improved the synbiotic fermentation efficiency of *U. pinnatifida* hydrolysates.

Keywords Synbiotic fermentation · γ-Aminobutyric acid · Fucose · *Lactobacillus brevis* · *Undaria pinnatifida*

Introduction

Brown seaweeds are high in easily degradable carbohydrates, making them suitable for synbiotic fermentation. The main carbohydrates are alginate, laminaran, fucoidan, and mannitol. Mannitol and glucose can be produced by hydrolysis of milled brown seaweed. Various physical, chemical, biological, and enzymatic pretreatments, and combinations thereof, have been used to increase hydrolytic yields [1]. For economic reasons, dilute acid hydrolysis is commonly used to prepare seaweed hydrolysates for enzymatic saccharification (*E*s) and fermentation.

Brown seaweed, which is a prebiotic, has received much attention in the fields of nutrition and biomedicine given its unique physicochemical properties [2]. Fucoidan is a

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fucose-based sulfated polysaccharide an biotic derived from brown algae with antibacterial, antiviral, anti-oxidant, anticoagulant, anti-inflammatory, antitumor, antithrombotic, antifibrotic, and immunomodulatory activities [3]. Sulfated polysaccharides include fucoidans (chains of L-fucose with sulfated ester groups) from brown seaweeds, agars, and carrageenans (sulfated galactans), from red seaweeds and ulvans (sulfated glucuronoxylorhamnans), and other sulfated glycans from green seaweeds [4]. Here, the brown seaweed *Undaria pinnatifida* served as a prebiotic ingredient after synbiotic fermentation.

Synbiotics, which are synergistic mixtures of pre- and pro-biotics, and postbiotics (preparations of inanimate microorganisms and/or their components that confer a health benefit to the host) have been used to treat several clinical conditions and improve health [5–7]. Synbiotic and postbiotic fermentation are promising options for preparing functional fermented foods.

Gamma-aminobutyric acid (GABA), which is a non-protein amino acid, is one of the major inhibitory neurotransmitters in the human central nervous system and is also widespread in animals, plants, and microorganisms [8]. However, most studies focused on GABA-producing microorganisms rather than GABA per se. GABA-producing lactic acid bacteria (LAB) include strains of *Lactococcus lactis*, *Lactobacillus* (*Lb.*) *brevis*, *Lb. buchneri*, *Lb. helveticus*, *Lb. paracasei*, *Lb. plantarum*, and *Streptococcus thermophilus* [9–15]. The biosynthetic production of natural GABA by LAB (for food manufacture) exploits the health-promoting properties of GABA and LAB, which are probiotics. Of the LAB species, *Lb. brevis* has shown great promise in terms of large-scale fermentation for GABA production [16]. Thus, we selected *Lb. brevis* as the optimal probiotic for GABA production. To further increase the yield of GABA, the effects of the pyridoxal 5′-phosphate (PLP) cofactor and initial monosodium glutamate (MSG) concentrations on GABA production by *Lb. brevis* were investigated using *U. pinnatifida* hydrolysates.

The objective was to optimize pretreatment and *E*s in terms of GABA production, and assess the synbiotic fermentation of synergistic mixtures of prebiotics and probiotics from *U. pinnatifida* hydrolysates.

Materials and Methods

Chemicals and Strains

All chemicals used were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Duksan Pure Chemical Co. (Gyeonggi-do, Korea). *Lb. brevis* KCL010 was obtained from the Korean Culture Collection of Probiotics (KCCP, Seongnam, Korea) and was cultured in Difco Lactobacilli de Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Co., Sparks, MD, USA) at 30℃ and 120 rpm for 18 h. Then, 5% (w/v, 2.8 g dcw/L) inoculates were transferred to 250-mL Erlenmeyer flasks containing 100-mL amounts of *U. pinnatifida* hydrolysate. To determine cell growth via optical density measurements, the hydrolysate was centrifuged at 8000x*g* for 15 min to eliminate solids. The supernatant served as the fermentation medium.

Seaweed Preparation

U. pinnatifida (Sea mustard, Miyuk) was obtained from Gijang Local Products Co. Ltd. (Busan, Korea). The seaweed was ground using a roller mill and sieved through a 200-mesh filter before pretreatment. Composition and proximate analyses of *U. pinnatifida*

were performed by the Feed and Foods Nutrition Research Center at Pukyong National University, Korea, using the AOAC method [17]. Then, the levels of carbohydrates and cellulose were employed to calculate the efficiencies of pretreatments and *E*s.

Thermal Acid Hydrolysis and Enzymatic Saccharification

Thermal acid hydrolytic pretreatment of *U. pinnatifida* was performed under various conditions, i.e., using seaweed slurry concentrations of 2–12% (w/v), thermal hydrolysis times of 15–120 min, and H_2SO_4 concentrations of 90–540 mM. The pH of *U. pinnatifida* hydrolysates was then adjusted to pH 5.0 with 5 N NaOH. The hydrolysates served as fermentation media (with 4% [w/v] MSG) for GABA production. The hydrolysates were fermented at 30°C and 150 rpm with *Lb. brevis* KCL010. The pretreatment efficiency (E_p, %) was calculated using Eq. (1) as the increase in mannitol and glucose concentrations ($\Delta S_{man+glu}$, g/L) during thermal acid hydrolysis relative to the total carbohydrate content of *U. pinnatifida* (TC, g/L).

$$E_p(\%) = \frac{\Delta S_{man+glu}}{TC} \times 100 \quad (1)$$

*E*s of the hydrolysates proceeded using Viscozyme L (β-glucanase, 121 U/mL; Novozymes, Bagsvaerd, Denmark), Celluclast 1.5 L (cellulase, 854 U/mL; Novozymes), and Spirizyme Fuel (a glucoamylase and cellulase blend, 862 U/mL; Novozymes). The three enzymes were diluted to 16 U/mL. Saccharification proceeded at 45°C and 150 rpm for 48 h after thermal acid hydrolysis. To achieve a synergistic effect (compared to single-enzyme treatments), the enzymes were mixed in a 1:1:1 ratio (16 U/mL of each enzyme).

The efficiency of E_s (%) was determined as the increase in glucose concentration (ΔS_{glu}, g/L) from the fiber level (F, g/L) during pretreatment, as follows:

$$Es(\%) = \frac{\Delta S_{glu}}{F} \times 100 \quad (2)$$

Determination of Fucoidan Content

The measurement methods were based on determination of common chemical features (e.g., fucose, sulfated polysaccharides, fuco-oligosaccharides) of fucoidan. The fucoidan content was determined by measuring the amount of fucose released from *U. pinnatifida* hydrolysates. The fucose was quantified using an enzymatic L-fucose assay kit (K-FUCOSE; Megazyme, Wicklow, Ireland) according to the manufacturer's instructions. The assay is based on oxidation of L-fucose to L-fucono-1,5-lactone by L-fucose dehydrogenase in the presence of nicotinamide adenine dinucleotide phosphate ($NADP^+$). The amount of L-fucose is equivalent to the level of reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced. The levels of NADPH were measured by optical absorbance. Briefly, 0.1 mL of reaction mix was added to 2.5 mL of mixed reagents (2.0 mL dH_2O, 0.4 mL reaction buffer [pH 9.5], 0.1 mL $NADP^+$, and 0.05 mL L-fucose dehydrogenase suspension). The mixture was held at room temperature for 20 min, after which the optical density was measured using a UV–visible spectrophotometer (EMC-18PC-UV; EMCLAB, Duisburg, Germany) at 340 nm. The blank was prepared as above, but 0.1 mL of distilled water was added instead of the sample supernatant (0.1 mL). The fucose concentration was calculated using the following Eq. (3):

$$\text{Fucose}(\text{g/L}) = 0.6905 \times \Delta A_{\text{L-fucose}} \quad (3)$$

Synbiotic Fermentation

Each synbiotic fermentation was performed using 100 mL of seaweed hydrolysate in a 250-mL Erlenmeyer flask under semi-anaerobic conditions. *E*s and final pH correction to pH 5.0 were performed after thermal acid hydrolysis. A seed culture of LAB was grown at 30°C and 120 rpm for 18 h in 30 mL of MRS medium and a 5% inoculate (w/v, 2.8 g dcw/L) transferred to a 250-mL Erlenmeyer flask containing 100 mL of *U. pinnatifida* hydrolysate. Nutrient supplements (2.0 g/L yeast extract, 5 g/L K_2HPO_4, 0.25 g/L $MgSO_4$, 10 μM PLP and 40 g/L MSG) were added to the hydrolysate and mixed before inoculation. Mannitol adaptation was performed using *Lb. brevis* KCL010 cultured in MRS medium with 80 g/L mannitol (MRSM) for 48 h. Then, the cells were washed twice with fresh MRS medium and transferred to the *U. pinnatifida* hydrolysate for synbiotic fermentation, after centrifugation at 1390 × *g*, 10 min to remove the MRSM medium. The seaweed hydrolysates were fermented at 30°C and 150 rpm with *Lb. brevis* KCL010. Samples were obtained periodically for determination of cell growth (OD_{600nm}) and GABA, mannitol, glucose, fucose, and MSG concentrations, and stored at –20°C before analysis. The efficiency of GABA conversion was calculated as follows:

$$\text{EGC}(\%, \text{w/w}) = \left[(\text{GABA})\text{inc}/(\text{MSG})\text{cons}\right] \times 100 \quad (4)$$

where (GABA)inc represents the increase in GABA concentration (g/L) and (MSG)cons is the consumption of MSG (g/L) during LAB fermentation.

Analytical Methods

Cell growth was monitored by measuring OD_{600} values using a UV–visible spectrophotometer (EMC-18PC-UV). Salinity was measured by a digital salinity refractometer (ES-421; ATAGO, Tokyo, Japan).

MSG and GABA contents were determined by pre-column derivatization with 2-hydroxynaphthaldehyde, followed by HPLC. The well-established derivatization procedure is detailed elsewhere [18].

Glucose and mannitol concentrations were determined using a high-performance liquid chromatography (HPLC) system (Agilent 1200 Series; Agilent Inc., Santa Clara, CA, USA) equipped with a refractive index detector. An Aminex HPX-87H column (300.0 × 7.8 mm; Bio-Rad, Hercules, CA, USA) was maintained at 65℃, and samples were eluted with 5 mM H_2SO_4 at 0.6 mL/min. The values are the means from triplicate experiments.

Results and Discussion

U. pinnatifida Composition and Thermal Acid Hydrolysis

The composition of *U. pinnatifida* was analyzed by the AOAC method and was as follows: 48.5% carbohydrate, 3.5% crude fiber, 18.2% crude protein, 1.8% crude lipid, and 28.0% crude ash.

The effects of the various slurry levels were determined at 121°C with a thermal hydrolysis time of 60 min and H_2SO_4 concentration of 360 mM [Fig. 1A]. The concentrations of monosaccharides, such as mannitol and glucose, increased with increasing slurry level. Notably, the glucose concentration increased only slightly when the slurry level increased to > 12% (w/v). The initial glucose concentration was 0.22 g/L after thermal acid hydrolysis pretreatment; this was considered when evaluating the efficiency of pretreatment (E_p). At a monosaccharide (mannitol and glucose) concentration of 7.59 g/L, the slurry content was 8% (w/v) with an E_p of 19.3% (g/g). However, a high slurry content increased the salinity (%, g/g) of pretreated slurry from 2.9 to 6.4%. GABA production decreased from 2.38 to 1.65 g/L as the slurry content increased from 8 to 12% (w/v). This indicated that high-salt stress of *Lb. brevis* KCL010 significantly impeded GABA production during the fermentation of thermal acid-hydrolyzed seaweed medium. Similar results were reported by Laroute et al. [19]; cell yield was reduced by growth inhibition at high chloride concentrations and, consequently, GABA production decreased. We thus employed a slurry content of 8% (w/v) in subsequent experiments.

The effects of thermal acid hydrolysis of 8% (w/v) slurry with 360 mM H_2SO_4 at 121°C for different times are presented in Fig. 1B. The maximum monosaccharide (including the initial glucose) concentration over a hydrolysis time of 30 min was 7.61 g/L with an E_p of 19.6%. However, the E_p after hydrolysis for 60–120 min was no greater than that after 30 min, possibly because 30 min was adequate to hydrolyze all sugars. Similar results have been reported in that, an extended hydrolysis time or a high temperature could have a negative effect on the monosaccharides yields [20]. Therefore, a hydrolysis time of 30 min was selected for subsequent experiments.

The effects of thermal acid hydrolysis of 8% (w/v) slurry at 121°C for 30 min under various H_2SO_4 concentrations are shown in Fig. 1C. The control group lacked acid. Thermal acid hydrolysis increased the monosaccharide concentrations at all sulfuric acid concentrations tested, and is optimal for digesting polysaccharides in *U. pinnatifida* hydrolysates. A further increase in the H_2SO_4 concentration to > 180 mM did not enhance monosaccharide degradation or GABA production. Redding et al. [21] reported that 0.9% and 1.2% acid showed the most options for different temperature and time combinations. Therefore, 180 mM H_2SO_4 was selected as a suitable acid level; the final monosaccharide concentration was 7.96 g/L and the E_p was 20.5%. In summary, the optimal thermal acid hydrolysis conditions were 8% (w/v) slurry, 180 mM H_2SO_4, and 121°C for 30 min.

Enzymatic Saccharification of U. pinnatifida

The effects of *E*s on glucose release from *U. pinnatifida* hydrolysate were investigated according to enzyme type [Fig. 2A] and dosage [Fig. 2)]. As shown in Fig. 2A, single treatments with Celluclast 1.5 L (C), Viscozyme L (V), and Spirizyme Fuel (S), with consideration of the initial glucose concentration, led to increases of 2.6 g/L glucose with an E_s of 93.1%, 2.1 g/L glucose with an E_s of 74.1%, and 1.5 g/L glucose with an E_s of 53.7% respectively. Mixed enzyme treatments with C+V, V+S, and V+S (1:1 ratio) had no synergistic effects compared to treatment with Celluclast1.5 L alone. Single-enzyme treatment of *U. pinnatifida* hydrolysate with Celluclast 1.5 L was preferable to mixed enzyme treatments. Next, *E*s proceeded over 24 h; we aimed to identify the optimal treatment time for obtaining a high glucose concentration. Celluclast 1.5 L was the optimal enzyme after pretreatment of fiber in *U. pinnatifida* hydrolysate. However, the opposite effect was observed

Fig. 1 The effects of seaweed, thermal acid hydrolysis for various times, and the acid concentration on pretreatment efficiency: **A** *U. pinnatifida* slurry content, **B** thermal hydrolysis time, and **C** H_2SO_4 concentration. After thermal acid hydrolysis and mixed enzyme (C+V, 1:1 ratio; 16 U/mL) pretreatment, the effect of *Lb. brevis* KCL010 fermentation on GABA production was analyzed. Conditions: 30°C, 150 rpm for 72 h, 4% (w/v) MSG

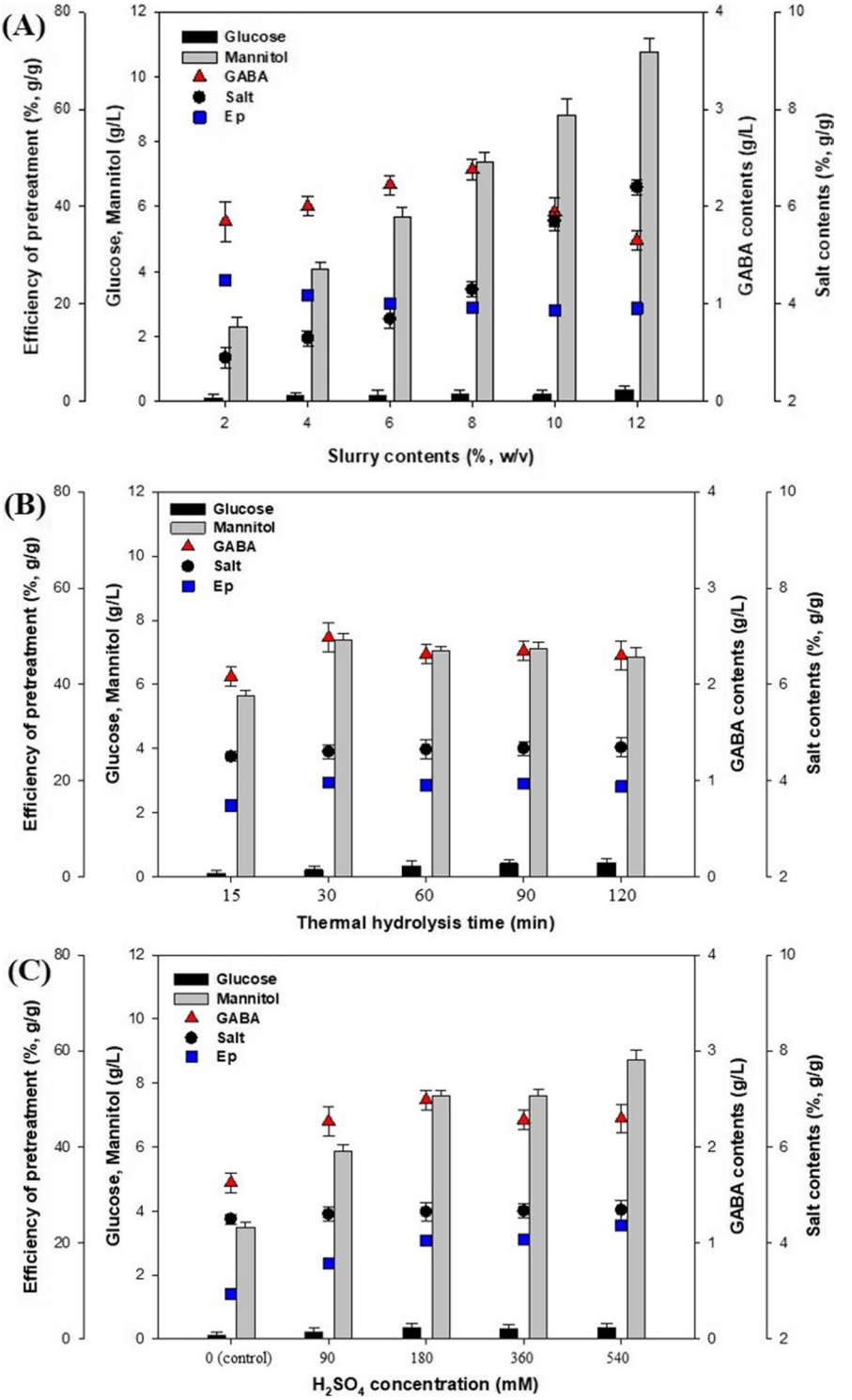
with saccharification of *Laminaria japonica* hydrolysates. Kim et al. [22] performed simultaneous saccharification and fermentation of an acid hydrolysate of *L. japonica* using a mixture of commercial enzymes (Celluclast 1.5L, Novoprime B969, and Viscozyme L). The *E*s process depended greatly on the algal species studied [23].

To determine the optimal dosage of Celluclast 1.5 L, saccharification proceeded with 8–32 U/mL enzyme at 45°C and 150 rpm for 24 h [Fig. 2B]. The optimal enzyme level was 8 U/mL; further increases to 32 U/mL had no significant additional effect on E_s. Thus, an enzyme dosage of 8 U/mL was selected for *E*s of the hydrolysate. In terms of prebiotic production, the fucose concentration was 0.48 g/L, and further increases in the enzyme level to 32 U/mL did not significantly increase it. Similar results were obtained in the seaweed composition analyses. Usov et al. [24] determined the Busov polysaccharide compositions of 17 species, particularly the fucoidan contents (calculated as twice the fucose contents, assuming that the average fucose content is 50% that of fucoidan). Several studies have reported that the brown seaweed *Ascophyllum nodosum* modulates the composition of the gut microbiota. The seaweed increased the relative abundances of Bacteroidetes and Firmicutes, suggesting potential utility as a functional food to improve human gut health [25, 26]. Therefore, *U. pinnatifida* hydrolysate may serve as a valuable prebiotic ingredient for synbiotic fermentation.

The Effects of MSG and PLP on GABA Production

MSG and PLP are required when GAD converts L-glutamate to GABA [27]. The effects of MSG (Fig. 3A) and PLP (Fig. 3B) addition to pretreated *U. pinnatifida* hydrolysate were investigated; the goal was to increase GABA production by *Lb. brevis* KCL010 during 72 h of fermentation. Figure 3A shows the GABA production by *Lb. brevis* KCL010 with and without MSG at 0–5% (w/v). Compared to the control group, GABA production generally increased with the MSG concentration; however, it decreased slightly between MSG addition amounts of 4 to 5% (w/v). The GABA production amount was 2.23 g/L, and the efficiency of GABA conversion (*E*GC) was 11.86% when *Lb. brevis* KCL010 grew in the presence of 3% (w/v) MSG; this concentration was optimal. Similar results were obtained for *Lactiplantibacillus plantarum* FBT215 [28]; GABA production (238.43 μg/mL) was maintained by 200 mM MSG (3.4%) in MRS broth; however, production decreased slightly when the MSG level was 250 mM in modified MRS broth. Villegas et al. [29] reported a gradual increase in GABA yield as the MSG level increased from 0 to 270 mM. Higher MSG concentrations decreased the GABA yield, possibly because an increase in osmotic pressure compromised bacterial metabolism.

Figure 4B shows that the addition of PLP had little effect on GABA production by *Lb. brevis* KCL010 acting on pretreated *U. pinnatifida* hydrolysate. GABA production barely increased; however, MSG consumption decreased with increasing PLP concentration. An increase in PLP concentration from 10 to 30 μM increased the E_{GC} from 12.3 to 15.1% but further increases in PLP concentration (up to 50 μM) had no significant additional effect on E_{GC}. Therefore, 30 μM PLP was optimal. Theoretically, PLP addition should increase GAD activity and GABA production: 20 μM PLP significantly enhanced GABA production and GAD activity on *S. thermophilus* Y2 culture [15]. In contrast, the addition of PLP to the fermentation medium of *L. plantarum* 90sk did not affect GABA production [30]. It


(A)
Glucose
Mannitol
GABA
Salt
Ep
Efficiency of pretreatment (%, g/g)
Glucose, Mannitol (g/L)
GABA contents (g/L)
Salt contents (%, g/g)
Slurry contents (%, w/v)
(B)
Thermal hydrolysis time (min)
(C)
0 (control)
H2SO4 concentration (mM)

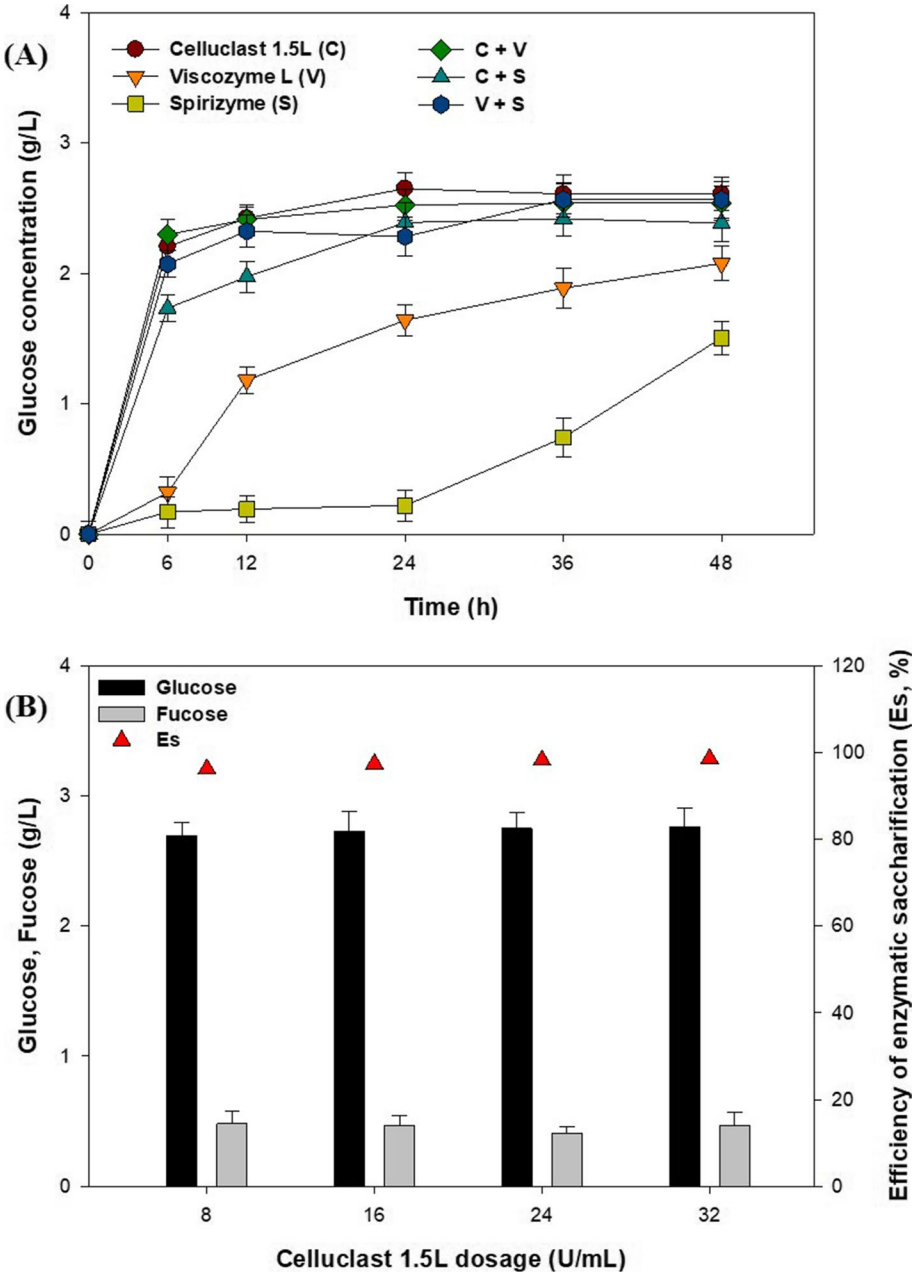


Fig. 2 The effects of **A** enzyme type and **B** dosage on glucose release from *U. pinnatifida* hydrolysate (an 8% [w/v] slurry at pH 5.0, 45°C, and 150 rpm) over 48 h after thermal acid hydrolysis. The initial glucose level was 0.22 g/L after pretreatment

is possible that PLP is degraded in the early phase of growth. Dependence on the timing of PLP addition was previously reported by Yang et al. [15]. Very small quantities of PLP in the culture medium may suffice for evoking glutamate decarboxylase (GAD) activity.

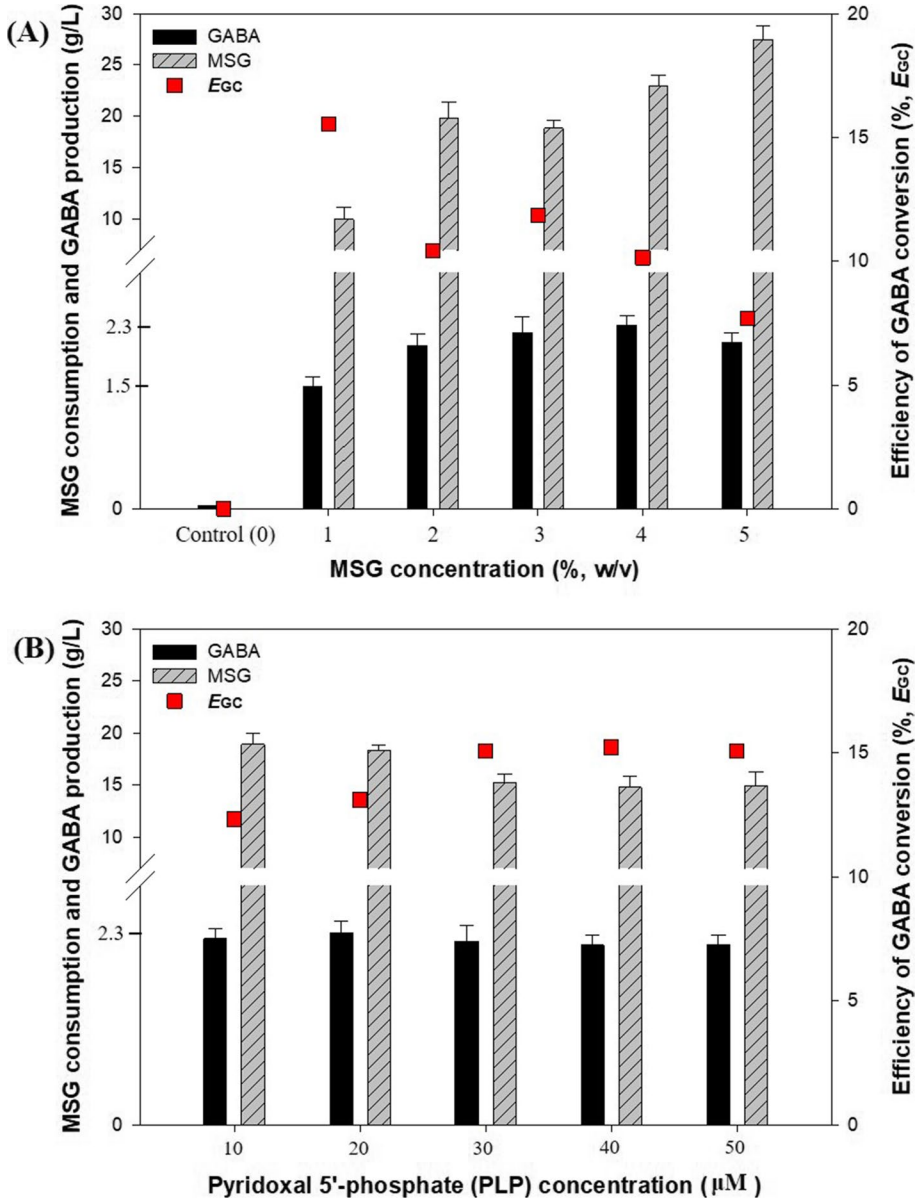


Fig. 3 The effects of **A** MSG and **B** PLP concentrations on GABA production by *Lb. brevis* KCL010

Synbiotic Fermentation with Non-adapted and Adapted Lb. brevis KCL010

Figure 4 shows synbiotic fermentation by *Lb. brevis* KCL010 adapted and not adapted to high mannitol concentrations. Figure 4A shows that all glucose was consumed within 48 h; however, the mannitol and MSG levels were not completely spent until 120 h. This probably reflects the preference of LAB for glucose compared to other monosaccharides;

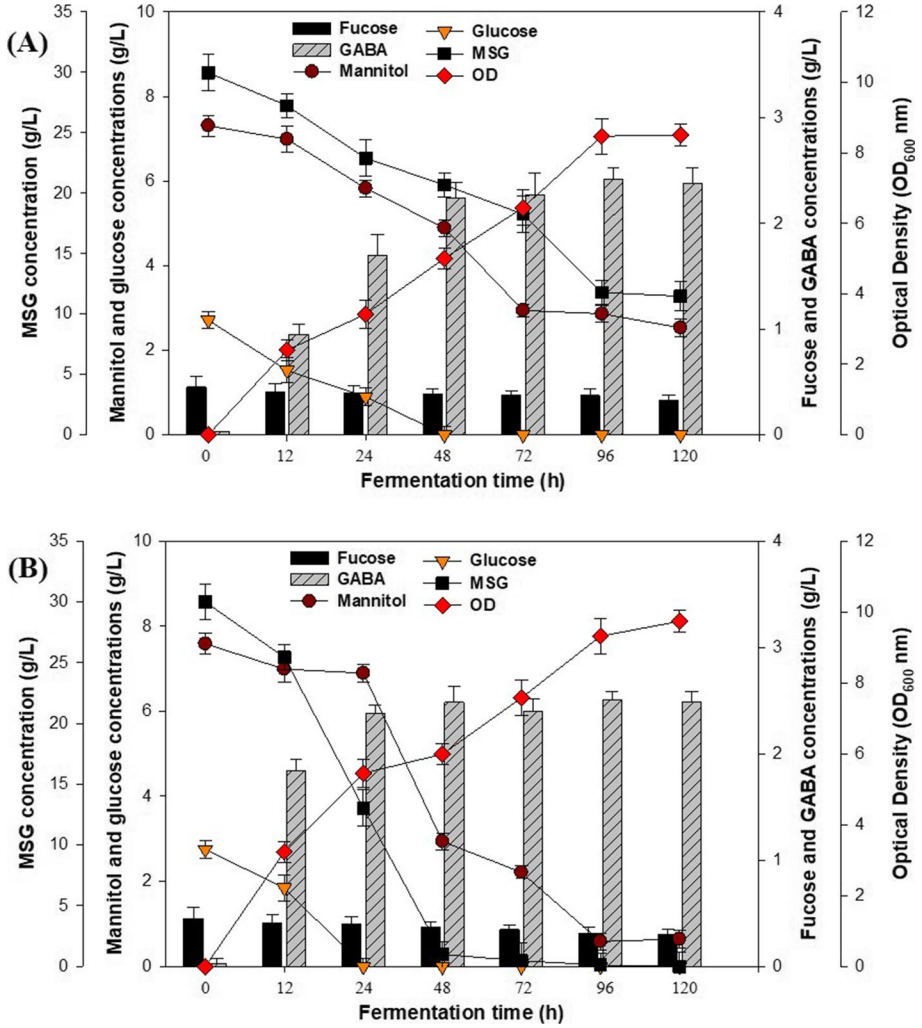


Fig. 4 Symbiotic fermentation of **A** non-adapted *Lb. brevis* KCL010 and **B** *Lb. brevis* KCL010 adapted to high mannitol concentrations. The tests were performed in 250-mL Erlenmeyer flasks with 100 mL amounts of *U. pinnatifida* hydrolysate

mannitol consumption is inefficient. This appears to be a strain-specific phenomenon [31]. Growth of *Lb. brevis* KCL010 with 30 g/L MSG exhibited no lag; the stationary phase was reached by 96 h. The GABA concentration after 120 h of fermentation was 2.37 g/L; the fucose concentration decreased slightly over time. However, an additional treatment is needed to solve the problems posed by carbon catabolite repression (CCR) (Fig. 4A). In the presence of both glucose and mannitol, repression by glucose seems to predominate over induction by mannitol. The current study strongly suggests that glucose represses the mannitol operon of LAB.

To overcome these problems, *Lb. brevis* KCL010 was adapted to high concentrations of mannitol, as shown in Figure 4B. Glucose was rapidly consumed within 24 h. Mannitol was consumed after 96 h; only 0.64 g/L of mannitol remained in the

fermentation medium. Compared to Fig. 4A, Fig. 4 B shows that glucose and mannitol were sequentially used to promote diauxic cell growth. The concentration of GABA was 2.50 g/L after 120 h of fermentation. Choe et al. [32] reported that mannitol operon repressor (MtlR)-histidine phosphocarrier protein (HPr) interactions are the key mechanism by which glucose represses the mannitol operon in *E. coli* (independent of the mannitol induction mechanism). Similar results were obtained in terms of glucose repression of xylose utilization by *Lactococcus lactis* IO-1; a mixture of glucose and xylose triggered diauxic cell growth, which was attributable to CCR [33]. *L. brevis* utilizes glucose and xylose in parallel [34, 35]. Thus, our adapted *Lb. brevis* KCL010 facilitated efficient utilization of both mannitol and glucose during synbiotic fermentation of *U. pinnatifida* hydrolysate.

Many factors affect the ability of lactic acid bacteria to produce GABA [14, 30]. Maintenance of high bacterial density may increase GABA yield via the production of GAD [36]. Although the *U. pinnatifida* hydrolysate is a synthetic medium appropriate for synbiotic production because of its low cost, and the fact that it can serve as a dietary supplement benefiting gut health, further improvements are needed to enhance the productivity of synbiotic fermentation. When both probiotics and prebiotics act together in the gastrointestinal tract, the synergistic effects are optimized. Synergistic interactions between prebiotics and probiotics enhance human health and metabolism [37, 38].

Conclusions

It has been optimized thermal acid hydrolysis pretreatment of the seaweed *U. pinnatifida* using various slurry levels, thermal hydrolysis times and H_2SO_4 concentrations. After pretreatment, i.e., during fermentation, high-salt stress of *Lb. brevis* KCL010 significantly impeded GABA production. The fucose concentration was 0.48 g/L after *E*s (assuming that the average fucose content is 50% that of fucoidan). Thus, *U. pinnatifida* hydrolysate may be a useful prebiotic. Various PLP concentrations had no significant effects on GABA production because PLP is degraded in the early phase of growth. Comparison between non-adapted *Lb. brevis* KCL010 and a strain adapted to high concentrations of mannitol showed that glucose repression could be overcome using adaptive evolution. Therefore, optimization of fermentation parameters may further improve the synbiotic fermentation of pre- and pro-biotics from *U. pinnatifida* hydrolysates.

Author Contribution Na Yeon Kim and Ji Min Kim designed the study and carried out the experimental part. Jong-Youn Son critically read and revised the manuscript. Chae Hun Ra supervised the work and experimental designs, and approved the final manuscript for submission. All authors read and approved the final manuscript.

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Declarations

Competing Interests The authors declare no competing interests.

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