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Influence of Extracellular Products from *Haematococcus pluvialis* on Growth and Bacteriocin Production by Three Species of *Lactobacillus*

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Abstract The effects of *Haematococcus pluvialis* extracellular products on microbial growth and bacteriocin production were investigated to improve bacteriocin synthesis during the growth cycle of Lactobacilli. *Lactobacillus pentosus* KJ-108, *L. plantarum* KJ-10311, and *L. sakei* KJ-2008 were cultured in MRS and enriched medium (ERM) with or without supplement of the extracellular products obtained from a late exponential phase culture of *Haematococcus pluvialis* in modified Bold's basal medium (MBBM). In both MRS and ERM, the extracellular products strongly enhanced the growth as well as the bacteriocin production of all the lactic acid bacteria tested. The enhancing effect was observed in ERM with pH adjusted at 5 and 6. In addition, some difference in growth effects with the extracellular products of *H. pluvialis* was observed between pH 5 and 6 in ERM, but no effect was observed in the minimal medium. The final biomass and the final concentration of bacteriocin activity were associated with the cell growth that was promoted by the extracellular products of *H. pluvialis*, and the enhanced cell growth of the three lactic acid bacterial strains induced the increase of the specific bacteriocin production. Therefore, bacteriocin production and activity were influenced by the addition of the extracellular products of *H. pluvialis* in the culture medium.

Key words: *Lactobacilli*, *Haematococcus pluvialis*, extracellular products, bacteriocin

Algae are great sources of many highly valuable products such as polyunsaturated fatty acid, β-carotene, astaxanthin, and other bioactive compounds. Therefore, they have been recognized for centuries as sources for human food, iodine, alginic acid, agar, and carrageen. Recently, blue-green and green algae have been cultured industrially and sold for health food, because of their high content of protein, vitamins, and other nutrient supplements. *Spirulina* and *Chlorella* are the best-known genera for successful commercialization because of their nutritional values. Currently, quite a few countries, especially in Asian-Pacific countries including Korea, are cultivating microalgae on large commercial scales.

More recently, *Dunaliella* and *Haematococcus* have increasingly received attention owing to their production of β-carotene and astaxanthin, respectively. These carotenoids can be used as a pigmentation source in the diets of farmed fish and animals [18] and have potential clinical applications due to high antioxidant activities [7, 9, 29]. Specifically, *Haematococcus pluvialis* has been intensively studied as a natural astaxanthin producer owing to its ability to accumulate the orange-red pigment astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione). The astaxanthin produced by *H. pluvialis* is a mostly esterified (3S, 3'S) enantiomer, which is considered as the most stable and efficiently absorbed form [8, 25, 31]. Some green microalgae have been used in order to either stimulate or inhibit microbial growth [10, 16, 23]. In addition, algal extracts of *Scenedesmus armatus* [20] or *Chlorella pyrenoidosa* [30] have also been used as stimulating agents on the growth and development of both higher and lower plant organisms.

Probiotic lactic acid bacteria are used mainly as food supplements with dairy products. Lactobacilli, general members of probiotics, are normally colonized in the gastrointestinal tract [21] and are involved in the protection against intestinal pathogens [28]. Bacteriocins produced by lactic acid bacteria have been generally regarded as safe for human consumption for thousands of years [2, 17, 22]. Accordingly, *Lactobacillus* can play a major role in health and may stimulate endogenous or exogenous human gut microbial flora [6]. In addition, probiotics can be substituted for antibiotic agents in farmed animals to prevent intestinal infections, promote growth rate, and enhance the efficiency of feed conversions because of bacteriocin production [15]. Usually, *Lactobacillus* species produce functional metabolites, such as bacteriocins, during fermentation

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[3, 11]. Hence, the aim of this study was to investigate both growth and bacteriocin production of *Lactobacillus pentosus*, *L. plantarum*, and *L. sakei* in a cell-free culture medium containing extracellular products from *H. pluvialis*. Because more bioactive substances were released in standard laboratory media compared with MRS medium or enriched medium (ERM), standard laboratory media was used for the cultivation of lactic acid bacteria.

MATERIALS AND METHODS

Microorganisms

The green microalga *Haematococcus pluvialis* UTEX 16 was obtained from the University of Texas Culture Collection of Algae (Austin, TX, U.S.A.). *Lactobacillus pentosus* KJ-108, *L. plantarum* KJ-10311, and *L. sakei* KJ-2008 were isolated from pig feces [13, 14].

Culture Media and Growth Kinetics

H. pluvialis was cultivated in modified Bold's basal medium (MBBM) [27] containing 246.5 mg of $NaNO_3$, 175.6 mg of KH_2PO_4, 74.9 mg of K_2HPO_4, 70 mg of $MgSO_4 \cdot 7H_2O$, 25 mg of $CaCl_2 \cdot 2H_2O$, 25 mg of NaCl, 5 mg of EDTA-FeNa, 49.7 mg of $NaHCO_3$, 11.13 mg of H_3BO_3, 8.83 mg of $ZnSO_4 \cdot 7H_2O$, 1.57 mg of $CuSO_4 \cdot 5H_2O$, 1.44 mg of $MnSO_4 \cdot 5H_2O$, 1.19 mg of $Na_2MoO_4 \cdot 2H_2O$, and 0.49 mg $Co(NO_3)_2 \cdot 6H_2O$ per 1 l of distilled water. The microalga was grown in 2-l bubble column photobioreactors containing MBBM, with 0.2 vvm flow rate for 5% CO_2 gas, under continuous light intensity of 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the column surface. Temperature and pH were maintained at 25°C and 7.0±0.5, respectively. For the growth experiments of lactic acid bacteria, minimal medium (MM) [14], MRS medium (Merck, Darmstadt, Germany), and enriched medium (ERM) were used. The MM contained 0.3 g/l of NH_4CL, 1.5 g/l of KH_2PO_4, and 7.9 g/l of $Na_2HPO_4 \cdot 7H_2O$, and the ERM included 5 g/l of casamino acids vitamin-free (Difco), 3 g/l of glucose, and 25 g/l of nutrient broth (Merck, Darmstadt, Germany).

Extracellular products of *H. pluvialis* UTEX16 were obtained from a culture in late exponential stage with 1–3×10^5 cells. To establish bioactivity of the extracellular products present in the culture medium, the biomass of *H. pluvialis* was separated by filtration through a nitrocellulose membrane (0.22 μm pores, Millipore Corp., Bedford, MA, U.S.A.) and kept at 4°C. Then, the pH was adjusted to 5.0 with 4 N HCl. The same amount (v/v) of filtrate was added to the medium, MBBM, MRS, or ERM, including two-fold concentration of each medium component As control, an equal volume of MBBM with pH adjusted to 5.0 was added to the double concentration medium.

Lactic acid bacteria were precultivated for 16 h at 37°C in MRS medium. The cultures were then inoculated in the above-mentioned media with or without the extracellular products of *H. pluvialis* to obtain 0.03–0.05 initial optical density and incubated for 24 h at 37°C under anaerobic culture conditions. During 24 h at constant time intervals, 20 ml samples were aseptically withdrawn to measure optical density (OD) at 660 nm, cell dry weight, and bacteriocin activity (AU) for calculation of specific bacteriocin production rate. Optical density was calculated by the following equation, because any extracellular products did not have any effect on absorbance at 660 nm (data not shown).

$$OD_{660}=OD_t-OD_0$$

where OD_t and OD_0 are instantaneous and initial optical density at 660 nm, respectively, and t is the time at points of sampling.

Bacteriocin Activity Assay

The lactic acid bacterial cells were removed by centrifugation at 13,400 ×*g* (centrifuge 5415R, Eppendorf, Hamburg, Germany) for 15 min at 4°C, and the pH of the supernatant was adjusted to 6.5 by 1 N NaOH, treated with catalase, and sterilized through a microbial filter (0.22 μm pore, Millipore Corp., Bedford, MA, U.S.A.). To assay bacteriocin activity, the cell-free supernatant was diluted two-folds with Ringer's solution (quarter-strength, Oxoid, Basingstoke, Hampshire, U.K.). Then, 50 μl of the diluted samples was spotted on indicator lawns: Indicator lawns were prepared by overlaying MRS agar (1.5%, w/v; Merck) with 3.5 ml of MRS soft agar (0.7%, w/v). This top agar was inoculated with 100 μl of a fresh culture of *L. delbruekii* subsp. *bulgaricus* as the indicator that was grown to an A_{660} of 0.35–0.4.

After incubation at 30°C for 24 h, arbitrary units activity of the bacteriocin (AU/ml) were determined as the reciprocal of the highest dilution showing inhibition of the indicator strain [1, 19]. Specific bacteriocin production rate ($R_{b/w}$) was calculated by the following equation [26].

$$R_{b/w}=\frac{(B-B_0)}{(W-W_0)\times t}$$

where B and B_0 are the instantaneous and initial bacteriocin activity (AU/ml), respectively; W and W_0 are instantaneous and initial cell dry weight, respectively; and t is the time points during exponential growth phase.

RESULTS AND DISCUSSION

Growth Kinetics of Lactobacilli

The lactic acid bacterial strains KJ-108, KJ-2008, and KJ-10311 need nutritional supplements to grow, such as amino acids, vitamins, nucleic acids, *etc*. Figure 1 shows that MM was not enough to induce the growth of Lactobacilli because of insufficiency of nutrient to propagate the lactic

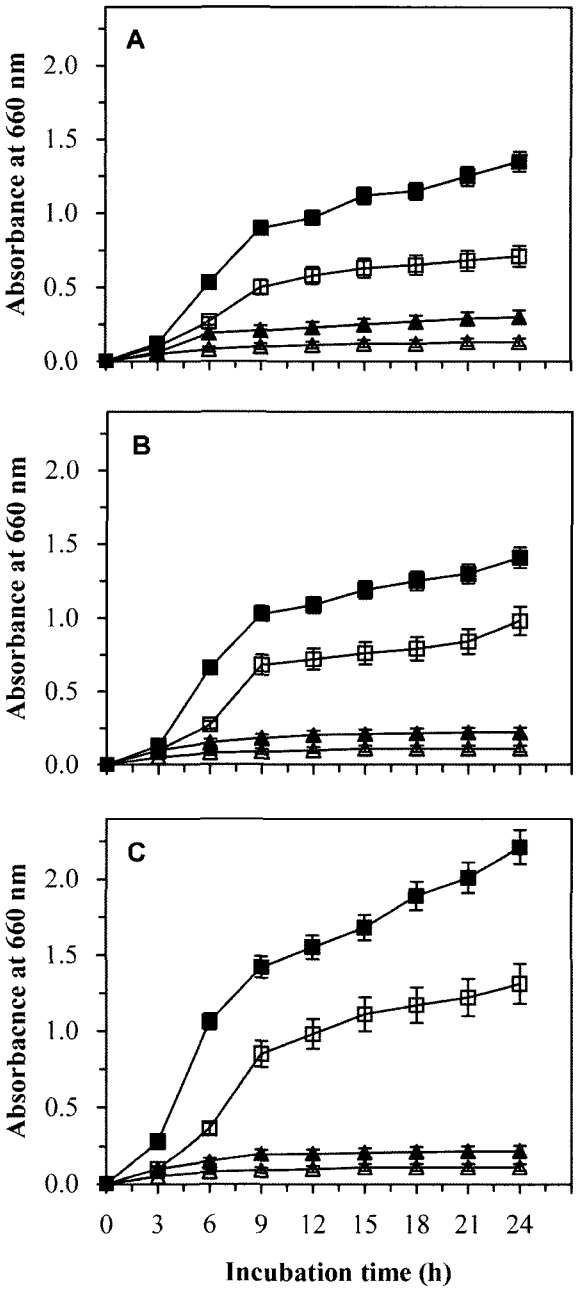

Fig. 1. The growth promoting effect of the *Haematococcus pluvialis* extracellular products on (**A**) *Lactobacillus pentosus* KJ-108; (**B**) *L. plantarum* KJ-10311; and (**C**) *L. sakei* KJ-2008 in minimal medium (MM) with (▲) or without (△) the extracellular products, and in MRS (pH 5) with (■) or without (□) extracellular products of *H. pluvialis*, not including the addition of any nutrient.

acid bacteria. Furthermore, the growth of Lactobacilli was promoted by the extracellular products of *H. pluvialis*, and MM contains low levels of the products. Moreover, enough inductions of the lactic acid bacterial cell growth were observed in MBBM, since MBBM (which is optimized for *Haematococcus* to enhance the production of astaxanthin) was not suitable for growth of the *Lactobacillus* species [27]. However, the growth of all the lactic acid bacterial strains examined in this work was strongly stimulated when they were inoculated into MRS medium to which cell-free filtrates of *H. pluvialis* were added (Fig. 1): When MRS was supplemented with *Haematococcus* filtrate, the growth of all the *Lactobacillus* strains examined was dramatically increased, whereas it was very slight in MM. In addition,

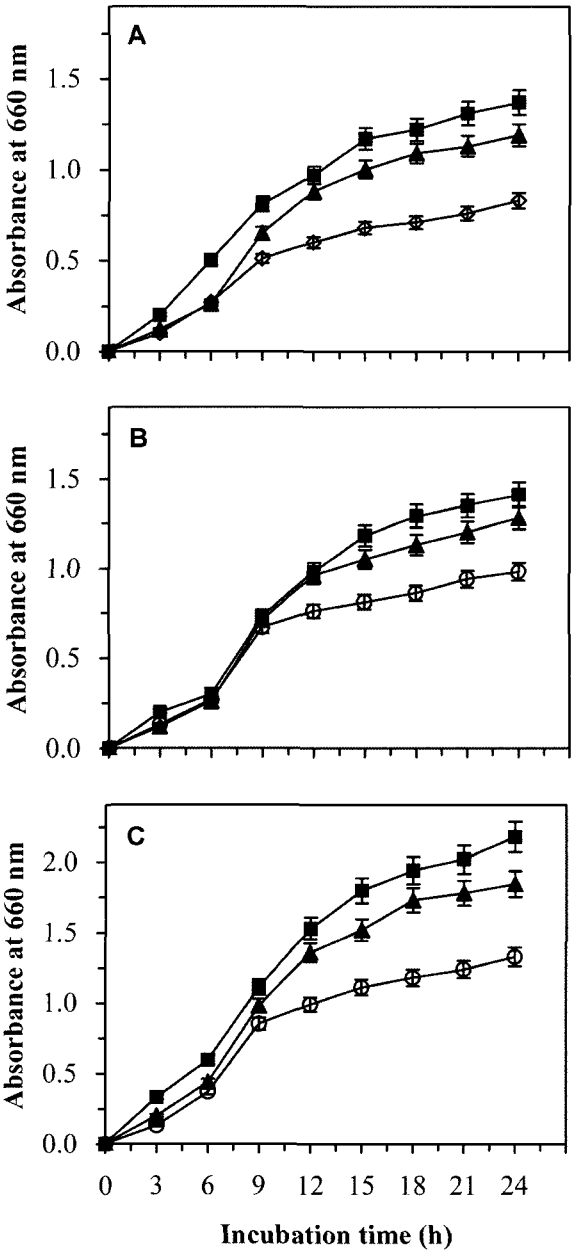

Fig. 2. The growth patterns of (**A**) *Lactobacillus pentosus* KJ-108; (**B**) *L. plantarum* KJ-10311; and (**C**) *L. sakei* KJ-2008 at pH 5 (■) or 6 (▲) in ERM with the extracellular product of *Haematococcus pluvialis*, and in MRS at pH 5 (○) without the extracellular product of *H. pluvialis*.

the responses to the extracellular products of *H. pluvialis* were so small that the effect of the extracellular product was negligible.

A series of experiments were carried out in order to find out the effect of *H. pluvialis* filtrates in ERM at two different pH values, 5 and 6. The growth was monitored spectrophotometrically in media with and without the addition of *Haematococcus* extracellular products and compared with growth in MRS medium of pH 5. Figure 2 shows significant increment of bacterial growth in all media enriched with extracellular filtrates, expressed as differential optical density (OD), compared with corresponding control medium. The extracellular products in ERM were found to stimulate the growth of the three Lactobacilli; 56.3–80.3% in *Lactobacillus pentosus* KJ-108, 20.4–43.9% in *L. plantarum* KJ-10311, and 9.9–63.4% in *L. sakei* KJ-2008, when compared with MRS without extracellular products.

To elucidate the basic composition of the cell-free filtrate of the algal culture medium, carbon, hydrogen, and nitrogen contents were investigated. For MBBM alone, carbon, hydrogen, and nitrogen contents were 5.0%, 1.7%, and 0.3% before the cultivation of *H. pluvialis*, and 11.5%, 8.9%, and 0.004% after its culture to the late exponential phase, respectively. *H. pluvialis* as a photoautotrophic microorganism consumes nitrogen in the culture medium and liberates some compounds that could be responsible for the stimulatory effect on growth of Lactobacilli [32]. Therefore, further studies will be focused on the isolation and characterization of these growth-promoting substances of the microalga.

The effect of *H. pluvialis* filtrates in ERM on the growth of lactic acid bacteria was investigated at different pHs, including 5 and 6. Figure 2 shows that the bacterial growth in all media supplemented with the extracellular filtrates significantly increased after 9 h of inoculation. Moreover, propagation of *L. pentosus* KJ-108, *L. plantarum* KJ-10311, and *L. sakei* KJ-2008 at pH 5 was slightly better than the propagation at pH 6. Significantly different growth effects were observed between MRS without the extracellular products of *H. pluvialis* at pH 5 and REM with the extracellular products at pH 5 and 6. Some difference in growth effect was observed between pH 5 and 6 in ERM with the extracellular products. Temperature had little effect on bacteriocin production of Lactobacilli (data not shown). However, pH has been reported to be one of the most important factors in bacteriocin production. Furthermore, the bacteriocin activity was more stable at acidic pHs than basic pHs: At pH 10 and higher, the bacteriocin activity was completely lost, whereas over 1,000 AU/ml of the bacteriocin activity remained at pH 1 [24]. According to the earlier studies [13, 14], the three lactic acid bacterial strains grow significantly faster at pH 5 than at pH 6. The different optical densities (OD) observed in the ERM containing the extracellular products of *H. pluvialis* might have been due to the effect of pH. Moreover, no significant difference in the growth of the three lactic acid bacterial strains was observed between MRS and ERM at pH 5, when the algal extracellular products were added (Figs. 1 and 2).

Kinetics of Bacteriocin Production

The different effects of the extracellular products of the microalga on the bacteriocin production by the three lactic acid bacterial strains are shown in Fig. 3. The bacteriocin

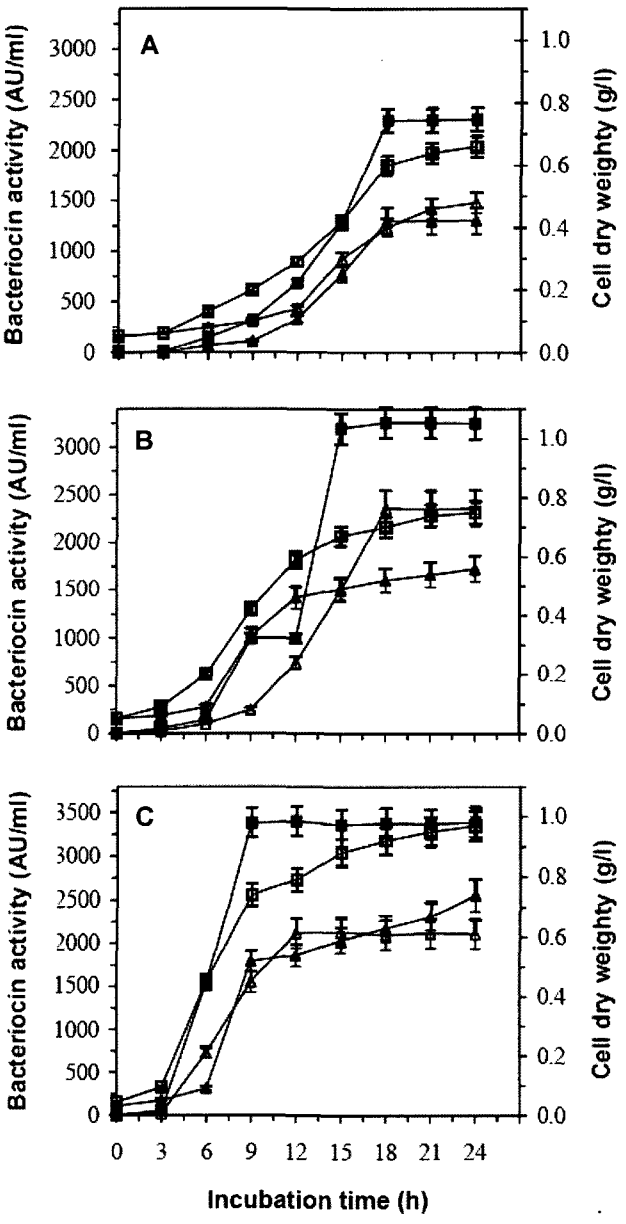

Fig. 3. The effect of the extracellular products on growth (solid symbols) and bacteriocin production (open symbols) of (**A**) *L. pentosus* KJ-108; (**B**) *L. plantarum* KJ-10311; and (**C**) *L. sakei* KJ-2008 in MRS with (■, □) and without (▲, △) the cell-free filtrate of *Haematococcus pluvialis* at pH 5.

production activity of the three lactic acid bacteria, KJ-108, KJ-10311, and KJ-2008, were similar. Bacteriocins were detected during the exponential growth phase, induced continuously throughout this phase, and reached a maximum level at the end of the exponential growth phase (or beginning of the stationary growth phase) [24]. These results suggest that bacteriocins exhibit main metabolic kinetics, indicating growth-related production [12]. Several studies [4, 26] show that some lactic acid bacteria produce bacteriocins during the exponential growth phase and their maximum activities are shown at the end of this phase or at the beginning of the stationary phase.

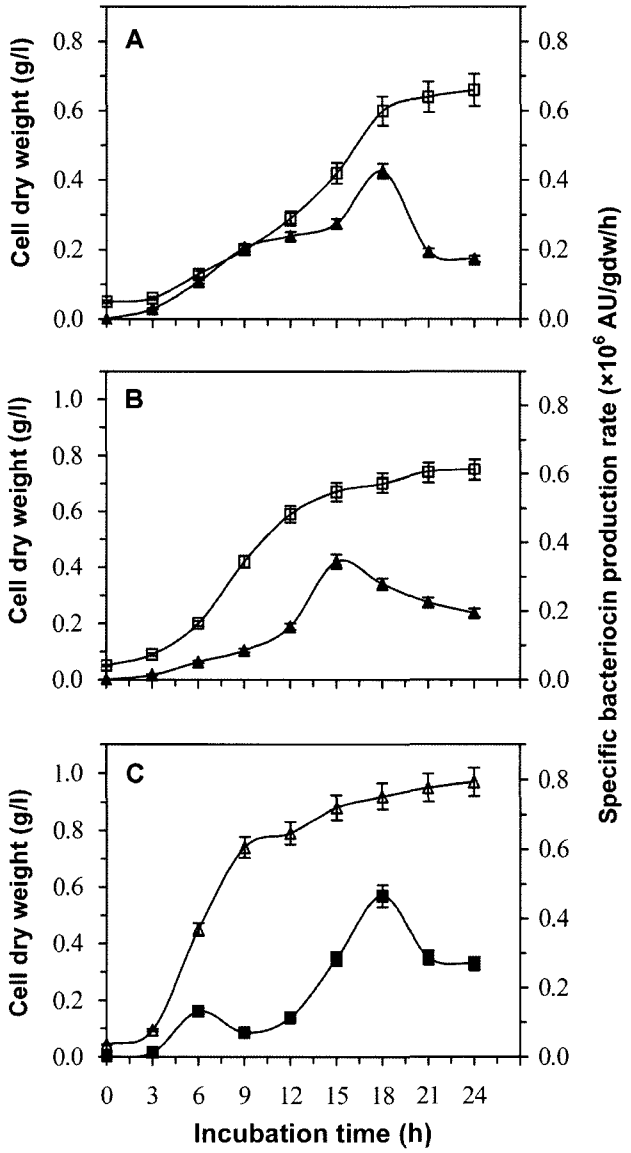

Fig. 4. The effect of the extracellular products of *Haematococcus pluvialis* on specific bacteriocin production rate during growth cycles of (**A**) *Lactobacillus pentosus* KJ-108; (**B**) *L. plantarum* KJ-2008; and (**C**) *L. sakei* KJ-10311 in MRS medium containing the extracellular product of *H. pluvialis* at pH 5 and 37°C.
Symbols: (□), cell dry weight; (▲), specific bacteriocin production rate. Values represent the mean of two experiments. The gdw stands for gram cell dry weight.

The results of the effect of the extracellular products of *H. pluvialis* on the specific bacteriocin production rate indicated different patterns of production of bacteriocin in batch culture among the three lactic acid bacteria tested (Fig. 4). Two major peaks in the specific bacteriocin production rate by *L. sakei* KJ-2008 were observed. The addition of the extracellular products of *H. pluvialis* resulted in enhancement of bacteriocin production, and the specific bacteriocin production rate occurred in three distinct phases. For the bacteriocins produced by *L. pentosus* KJ-108 and *L. plantarum* KJ-10311, the specific bacteriocin production rates were similar, and a slight increase of the final cell dry weight and the increase of the bacteriocin activities might be closely correlated with each other. For the bacteriocin produced by *L. sakei* KJ-2008, there was a little correlation between bacteriocin activity and cell dry weight (data not shown). Therefore, conditions favoring a strong increase of cell biomass would probably improve the bacteriocin concentration.

The results in the present study showed that the extracellular products of *H. pluvialis* stimulate the growth and bacteriocin production of the three test lactic acid bacteria *in vitro*, suggesting the possibility of use as probiotics in animal feed for improvement of lactic acid bacteria colonization in the intestinal tract. Therefore, further study will be focused on carrying out experiments in rats or animals to examine whether this food supplement may be used as a type of prebiotic [5]. Investigation of the effect of the *H. pluvialis* extracellular products on growth of intestinal pathogens is also required.

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