J. Gen. Appl. Microbiol., **56**, 481–489 (2010)

Full Paper

Conversion of squid pen by a novel strain *Lactobacillus paracasei* subsp. *paracasei* TKU010, and its application in antimicrobial and antioxidants activity

Tzu-Wen Liang,[1,2] Ying-Ying Wu,[2] Tsai-Yi Huang,[2] Chun-Yuan Wang,[2] Yue-Horng Yen,[3] Chin-Pei Liu,[2] Yan-Cheng Chen,[2] and San-Lang Wang[1,2,*]

[1] *Life Sciences Development Center and* [2] *Department of Chemistry, Tamkang University, Taipei 25137, Taiwan*
[3] *Department of Bioindustry Technology, Da-Yeh University, Chanhwa 515, Taiwan*

(Received June 11, 2010; Accepted September 30, 2010)

TKU010 was isolated from infant vomited milk and identified as *Lactobacillus paracasei* subsp. *paracasei*. TKU010 had desirable properties concerning its ability to withstand adverse conditions in the gastrointestinal tract. The hydrolysate of casein enhanced the growth of TKU010 most obviously (17.20–18.25 OD_{660}), followed by the hydrolysate of SPP (16.00–15.06 OD_{660}). Incubating with SPP, both the culture supernatant of TKU010 on the first day and the fourth day showed inhibitory activities on *E. coli* BCRC13086, *F. oxysporum* BCRC32121 and *A. fumigatus* BCRC30099. TKU010 culture supernatant (1% SPP) incubated for 3 days has high antioxidant activity; the DPPH scavenging ability was 75% per ml. Thus, TKU010 could be preferably used as a starter to produce fermented milk with possibly interesting organoleptic properties. Besides, we have shown that squid pen wastes can be utilized to generate a high value-added product, and have revealed its hidden potential in the production of biocontrol agents and functional foods.

Key Words——antimicrobial; antioxidant; *Lactobacillus paracasei*; probiotic ; squid pen

Introduction

The application of probiotic bacteria in food products is increasing due to potential health benefits associated with the consumption of these bacteria (Ong et al., 2007). *Lactobacillus* is the predominant genus of lactic acid bacteria found in milk samples. Fermented milk products containing viable lactobacilli have been used by humans primarily as a prophylactic aid and their use has been extended for treatment of intestinal infections (Xanthopoulos et al., 2000). Thus, the basic conditions for lactic acid bacteria to be used as probiotics include the following: (i) they should survive and grow in the intestinal tract, (ii) be safe, (iii) maintain viability during processing and storage, (iv) be tolerant to acid and bile, and (v) produce organic acids (Hyronimus et al., 2000; Lin et al., 2006).

It is well known that bioactive peptides from different sources of proteins have immunomodulatory effects, and antimicrobial and antioxidant activity (Gibbs et al., 2004; He et al., 2006; Kristinsson and Rasco, 2000). However, squid pen is a cheap protein/chitin-containing waste of marine processing. To further enhance the utilization of marine wastes, we have recently investigated the bioconversion of marine wastes for the production of proteases and/or chitinases (Wang and Yeh, 2006; Wang et al., 2006, 2008a, c). In a previous study, we found *L. paracasei* subsp. *paracasei* TKU010 and TKU012, two strains isolated from infant vomited

* Address reprint requests to: Dr. San-Lang Wang, Life Science Development Center, Taipei 25137, Taiwan.
Fax: +886 2 8631 1015
E-mail: sabulo@mail.tku.edu.tw (S.L. Wang)

milk, displayed protease activity and the ability to enhancing the growth of vegetables when cultured in a squid pen powder medium (Wang et al., 2008a; Wang et al., 2008c). The purpose of the present investigation was to study, in vitro, survival of TKU010 and TKU012 when exposed to low pH, human gastric juice, bile, and lysozyme, as well as their ability to grow in milk. The results would help us to evaluate the test strains concerning their above mentioned characteristics and select suitable cultures. Because of the favorable safety aspects of *L. paracasei,* we think that bioactive peptides produced by the proteases from lactobacilli are commercially acceptable. The antimicrobial and antioxidant physiological activities of the culture supernatants were also investigated.

Materials and Methods

Materials. The squid pen powder (SPP) used in these experiments was prepared as described earlier (Wang et al., 2008a). Squid pen was purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). In its preparation, the squid pen was washed thoroughly with tap water and then dried. The dried materials obtained were milled to powder for use as the carbon source for bioactive materials production. Casein was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potato dextrose agar (PDA), potato dextrose broth (PDB), MRS agar, and MRS broth were purchased from Difco Laboratories (Detroit, MI, USA). All other reagents used were of the highest grade available.

Strains and growth media. The test strains of *L. paracasei* subsp. *paracasei* TKU010, *L. paracasei* subsp. *paracasei* TKU012, and *L. rhamnosus* YQ101 had been isolated from infant vomited milk. *L. paracasei* subsp. *paracasei* BCRC12193, *L. kefir* BCRC14011, and *E. coli* BCRC13086 were obtained from Bioresource Collection and Research Centre (BCRC), Food Industrial Research Institute, Shin Chu, Taiwan. *L. paracasei* subsp. *paracasei* TKU012, *L. rhamnosus* YQ101, *L. paracasei* subsp. *paracasei* BCRC12193 and *L. kefir* BCRC14011 were used as the control strains. Strains were grown at 37°C in liquid or solid MRS medium (Difco Laboratories). Cultures were maintained in MRS broth with 20% sterile glycerol and stored at −80°C. The test fungus, *Fusarium oxysporum* BCRC32121, was kindly supplied by Dr. C.T. Lo, Department of Plant Pathology, Taiwan Agricultural Research Institute, Taichung, Taiwan. Another test fungus, *Aspergillus fumigatus* BCRC30099, was generously provided by Professor K.J. Tsai, Department of Food Science, National Taiwan Ocean University. Strains were grown at 25°C in PDA or PDB medium (Difco Laboratories). Cultures were maintained in PDB with 20% sterile glycerol and stored at −80°C.

Acid tolerance. Activated cultures were grown separately in MRS broth at 37°C overnight, and centrifuged (3,000×*g*, 10 min, 4°C). The cell pellet was washed once in sterile phosphate buffered saline (PBS, NaCl 0.8%, 0.1 M; pH 7) and subcultured in fresh MRS broth adjusted to different pH values (2, 2.5 and 3) with hydrochloric acid (0.1 M). The initial bacterial concentration was 10^7 CFU/ml and was checked by viable count determination on MRS broth as described below. Samples were incubated for 3 h at 37°C. Cells were serially diluted 10-fold in phosphate buffer (0.1 M, pH 7) in order to neutralize the medium acidity (Hyronimus et al., 2000). The residual viable count was determined by dilution and plate counting on MRS agar after 48 h of incubation. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial bacterial concentration.

Bile tolerance. Effects of bile on the growth of TKU010 and TKU012 were evaluated out by a method modified from Gilliland et al. (1984). MRS broth was inoculated with 10^6 CFU/ml from overnight cultures. Activated cultures were inoculated (1%) into MRS broth without or with 0.3% (w/v) oxgall (Sigma, LA, USA) and incubated at 37°C for 0, 3, 6, 9, 12, and 24 h with shaking at 80 rpm. Growth in control (no bile) and test cultures (0.3% oxgall) was measured by the absorbance of the cultures at 660 nm using a spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA). Growth curves were plotted and analysis was based on the time required for each culture. The bile tolerance of the sample without oxgall (control) was taken as 100%. Each assay was performed in triplicate.

Survival rates under simulated gastrointestinal conditions. The tolerance of lactobacilli under the simulated gastrointestinal conditions was determined using a previous method with minor modification (Lin et al., 2006). Activated cultures (0.2 ml corresponding to 10^6 CFU/ml) were mixed with 19.8 ml of sterilized PBS solution (pH 2.5). Each mixture was incubated at 37°C for 3 h with shaking at 80 rpm. After incubation, the survival lactobacilli were collected by centrifugation (7,000×*g*, 5 min) and washed once with PBS (pH 7).

They were resuspended in 10 ml MRS broth with or without 0.3% (w/v) oxgall bile and incubated at 37°C for 0, 3, 6, 9, 12, and 24 h. Bile tolerance of the lactobacilli was determined by measuring absorbance at 660 nm using a spectrophotometer (Bio-Rad). The bile tolerance of the sample without oxgall (control) was taken as 100%. Each assay was performed in triplicate.

Measurement of total acidity. Titratable acidity was determined according to the method of Chang et al. (2000). The pH of the samples was measured with a pH meter. Total acidity expressed as percent lactic acid was determined by titrating the culture supernatant with 0.1 M NaOH to pH 8.2.

Effect of cold storage on cell viability. Activated cultures (0.2 ml corresponding to 10^6 CFU/ml) were added to 10% of the milk drink diluted with sterilized PBS solution (pH 7). To examine the effect of cold storage on the cell viability of TKU010 and TKU012, the samples were stored at 4°C and 25°C for 4 weeks. Samples were taken at weekly intervals, and measured for the cell viability, the pH and the titratable acidity.

Resistance to lysozyme. MRS broth (10 ml) without and with lysozyme (60 U/ml) was inoculated (1%) by activated cultures and incubated at 37°C with shaking at 80 rpm (Xanthopoulos et al., 2000). Cells were serially diluted and growth was measured at 0 and after 2 h on MRS agar plates at 37°C for 48 h.

Effect of growth on other prebiotic substrates. MRS broth base free of fermentable carbon/nitrogen sources was used to investigate the ability of *L. paracasei* subsp. *paracasei* TKU010 to grow on prebiotic peptides. The tested peptides were added to the medium at 2.5% (v/v). The prebiotics investigated were the hydrolysates of casein and SPP hydrolyzed by *Bacillus subtilis* TKU007 protease (Wang and Yeh, 2006) and bromelain, respectively. Additionally, growth of this strain was examined on casein (Sigma). The organism was grown in triplicate experiments in 10 ml culture tubes under anaerobic conditions at 37°C for 12–96 h. The growth of the bacteria was monitored throughout the fermentations by measuring the culture O.D. at 660 nm.

In vitro antibacterial activity tests. The squid pen waste was fermented under optimized conditions as reported previously (Wang et al., 2008a, c). The fermented broth was centrifuged (4°C and 8,200×*g* for 20 min) and the supernatant was stored at 4°C. Antibacterial activity of the culture supernatant was assayed as follows: the culture supernatants were prepared and added to 5 ml of nutrition broth (NB, Difco Laboratories) to give a final concentration of 5% (v/v). To the above solutions, 0.05 ml of each bacterium (10^5 CFU/ml) was added and then incubated at 30°C for 0–4 days with shaking at 150 rpm. After incubation, viable cells were estimated by measuring the optical density at 660 nm.

In vitro antifungal activity tests. The antifungal activities of the culture supernatants were estimated using a growth inhibition assay described earlier (Wang et al., 2002). Fungal spores were grown on potato dextrose agar (PDA) in Petri plates. After 10 days of incubation at 25°C, the fungal spores were removed with sterile water containing 0.1% (v/v) Tween 80. The resulting suspension was filtered aseptically through sterilized gauze. The concentrations of the spore suspensions were determined in a hemacytometer and adjusted to 1×10^6 spores/mL. The spore suspensions were stored at 4°C before use. To test the antifungal inhibitory effect of the culture supernatants, Petri plates were filled with 5 ml of molten PDA precooled to 45°C and divided into two groups (triplicate for each). To each plate in the experimental group (E), the culture supernatant (5 ml) was added. The ratio (v/v) of the tested solution and PDA added in the Petri plates was 1 : 1. To those of the control group (C), an equal amount of sterile water instead of tested solution was added. After the plates had been cooled, the fungal spores (20 μl) were placed onto the agar surface. Both groups were incubated for 72 h at 25°C. The diameters of the largest and smallest fungal colonies were recorded and their averages were calculated. The inhibition ratios were calculated with the formula:

$$\text{inhibition ratio (\%)} = (C-E)/C \times 100$$

where C is the average diameter of the largest and smallest colonies of the control groups and E is the average diameter of the largest and smallest colonies of the experimental groups. Generally if the inhibitory ratio was >20%, the tested fungus was considered to be inhibited. To express the inhibitory activity of the culture supernatants, one unit of antifungal activity was defined as the amount of culture supernatant required to obtain a 50% inhibition under the above assay conditions.

Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals. The culture supernatant (150 μl) was mixed with 37.5 μl of methanolic solution containing 0.75 mM DPPH (Sigma) radicals. The mixture was shaken

vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank (Shimada et al., 1992). The scavenging ability was calculated as follows: Scavenging ability (%) = [(ΔA_{517} of control−ΔA_{517} of sample)/ΔA_{517} of control] × 100.

Results and Discussion

Taxonomic characterization of the TKU010 and TKU012 strains

Based on the results of our preliminary experiment, TKU010 and TKU012 are gram-positive non-spore forming bacilli, without catalase, oxidase, and mobility, which grow in both aerobic and anaerobic environments. According to the 16S rDNA partial base sequence, TKU010 and TKU012 are most similar to *Lactobacillus paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans*, with a similarity higher than 99%. However, according to the API identification system, TKU010 and TKU012 are most similar to *L. paracasei* subsp. *paracasei* but have 15 test items different from *L. paracasei* subsp. *tolerans* (data not shown). From above results, TKU010 and TKU012 should be *L. paracasei* subsp. *paracasei*.

Acid tolerance

The viable counts of *L. paracasei* subsp. *paracasei* TKU012 and *L. rhamnosus* YQ101 decreased rapidly after 3 h in phosphate-buffered saline, pH 2.0−3.0 at 37°C (Table 1). However, *L. paracasei* subsp. *paracasei* TKU010 was not affected under the same conditions. TKU010 showed a better resistance to low pH than TKU012 and YQ101. Survival rate of the test strains ranged between 39.7% (*L. rhamnosus* YQ101) and 87.2% (*L. paracasei* subsp. *paracasei* TKU010). The *L. paracasei* strains showed significant difference in their ability to withstand low pH. The survival rate of strain TKU010 was 83.8%, while that of strain TKU012 was only 42.3% at pH 2.0 for 3 h. Conway et al. (1987) observed three *L. paracasei* subsp. *paracasei* (strains DC411, DC412 and DC416) and one *L. rhamnosus* strain (DC425) remained almost unaffected by low pH after 2 h. In general, the strains of these two species (*L. paracasei* subsp. *paracasei* and *L. rhamnosus*) showed a better resistance to low pH than all the other tested strains (Conway et al., 1987). However, in this study, *L. paracasei* subsp. *paracasei* TKU012 and *L. rhamnosus* YQ101 were inhibited in low pH. As for acid tolerance of lactic acid bacteria, Gupta et al. (1996) observed that only two out of seven *L. acidophilus* strains tested exhibited growth at pH 3.0, while the results of Suscovic et al. (1997) on a *L. acidophilus* strain suggested a high acid tolerance at pH 3.0. Conway et al. (1987) also observed a better survival rate of *L. acidophilus* than *L. bulgaricus* at low pH.

Bile tolerance

Bile resistance is important for an organism that is expected to grow in the intestinal tract. In addition, bile resistance is an important characteristic to consider in selection of a culture as a dietary adjunct (Xanthopoulos et al., 2000). Variations existed among the cultures with regard to the growth of *Lactobacillus* strains in the presence of 0.3% bile (oxgall). Results showed that *L. paracasei* subsp. *paracasei* TKU012 (91.8% survival rate) was, in general, more resistant than strains of *L. paracasei* subsp. *paracasei* TKU010 (55.4% survival rate) and *L. rhamnosus* YQ101 (84.1% survival rate) at 0.3% bile for 3 h. However, the survival rates of the tested strains were almost unaffected at 0.3% bile for 24 h. The results were different from the results of Xanthopoulos et al. (2000). They found that *L. paracasei* subsp. *paracasei, L. acidophilus, L. gasseri, L. rhamnosus* and *L. reuteri* were inhibited in the presence of 0.15% bile for 24 h. Compared with the results of Xanthopoulos et al. (2000), TKU012, TKU010 and YQ101 exhibited a better resistance to bile.

Table 1. Acid tolerance of *Lactobacillus* strains incubated in MRS broth with different pHs.

Strains	Viable counts (CFU/ml)				Survival (%)			
	0 h	3 h			0 h	3 h		
	pH 7.0	pH 2.0	pH 2.5	pH 3.0	pH 7.0	pH 2.0	pH 2.5	pH 3.0
L. paracasei TKU010	$7.6\pm0.21\times10^8$	$2.8\pm0.10\times10^7$	$4.2\pm0.12\times10^7$	$5.6\pm0.16\times10^7$	100	83.8	85.8	87.2
L. paracasei TKU012	$7.2\pm0.22\times10^7$	$2.1\pm0.09\times10^3$	$1.2\pm0.05\times10^4$	$2.1\pm0.10\times10^5$	100	42.3	51.8	67.9
L. rhamnosus YQ101	$4.5\pm0.12\times10^7$	$1.1\pm0.08\times10^2$	$1.8\pm0.08\times10^3$	$3.2\pm0.10\times10^4$	100	39.7	42.5	58.8

All data are expressed as mean±SD from three different experiments (each experiment was conducted in triplicate).

Resistance tests under simulated gastrointestinal conditions

The simulated gastrointestinal conditions in animals are also an important factor which affects the lactobacilli viability (Gilliland and Walker, 1990). Although the composition of human bile juice is not exactly the same as that of the 0.3% oxgall solution, most studies used oxgall as one substitute for human bile because of their similarity (Brashears et al., 2003; Chou and Weimer, 1999; Gilliland and Walker, 1990; Lin et al., 2006; Noh and Gilliland, 1993). In this study, lactobacilli were cultured in MRS broth adjusted to pH 2.5 with or without 0.3% oxgall so that its tolerance to simulated gastrointestinal conditions could be evaluated. After 24 h of cultivation, effects of simulated gastrointestinal condition on the growth of LAB were observed. Results showed that *L. paracasei* subsp. *paracasei* TKU012 (90.5% survival rate) and *L. paracasei* subsp. *paracasei* TKU010 (89.3% survival rate) were, in general, more resistant than *L. rhamnosus* YQ101 (81.3% survival rate) under simulated gastrointestinal conditions. *L. paracasei* subsp. *paracasei* TKU012 and TKU010 showed better tolerance for simulated gastrointestinal conditions than *L. rhamnosus* YQ101. However, the survial rates of the tested strains were higher than 80% and were almost unaffected under simulated gastrointestinal conditions.

Resistance to lysozym

Lysozyme is capable of lysing certain bacteria, but does not significantly impair activities of lactic acid bacteria. Nevertheless, response to lysis may be extremely variable among strains and some species and resistant strains are not influenced by lysozyme (Xanthopoulos et al., 2000). Our results indicated that lactobacilli isolates from infant vomited milk showed a high resistance to lysozyme at 60 U/ml and was completely unaffected. Similar results have also been observed for some other strains such as *L. acidophilus* DC601, *L. rhamnosus* DC425, DC426, DC428 and *L. reuteri* DC420, DC423 (Xanthopoulos et al., 2000). Only *Lactobacillus acidophilus* strain DC602 showed a high degree of sensitivity in the presence of lysozyme (Xanthopoulos et al., 2000).

Effect of cold storage on cell viability

Factors affecting the viability of probiotic cultures include acidity (pH), oxygen level, and lack of nutrients in the product. It is important to have a significant number of viable lactic acid bacteria present in the finished product for maximum health benefits. Microencapsulation technology is being utilized to coat probiotic bacteria to extend shelf life, increase heat resistance and enhance acid resistance (Kyung et al., 2005). Table 2 illustrates the effect of cold storage on the viability of two lactic acid bacteria in milk or skim milk that had been stored for 4 weeks at 4°C. Although the lactic acid cultures in milk or skim milk gradually lost their cell viability during cold storage at 4°C, the viable cell counts of TKU010 in milk or skim milk still remained at 10^6–10^8 CFU/ml after 4 weeks of cold storage at 4°C. However, the viable cell counts of TKU012 in milk gradually fell during cold storage at 4°C. Similar results have also been observed in some other strains such as *L. casei*, *L. plantarum* and *L. debrueckii*. However, the viable cell counts of *L. acidophilus* were lower than others (Kyung et al., 2005).

Enhancement of growth

In previous studies, we had found that the extracellular proteases were produd by lactobacilli using squid pen as the sole carbon/nitrogen source (Wang

Table 2. Effect of cold storage on the viability of *L. paracasei* TKU010 and TKU012 lactic cultures.

Time (week)	In milk						In skim milk					
	TKU010			TKU012			TKU010			TKU012		
	pH	Acidity (%)	Viable counts (CFU/ml)	pH	Acidity (%)	Viable counts (CFU/ml)	pH	Acidity (%)	Viable counts (CFU/ml)	pH	Acidity (%)	Viable counts (CFU/ml)
0	6.45	0.12	$7.0\pm0.32\times10^5$	6.26	0.17	$2.9\pm0.14\times10^6$	6.53	0.10	$3.1\pm0.12\times10^5$	6.24	0.16	$3.9\pm0.18\times10^5$
1	5.50	0.22	$3.3\pm0.12\times10^7$	6.13	0.24	$1.7\pm0.08\times10^7$	5.48	0.28	$2.4\pm0.12\times10^7$	6.10	0.27	$2.3\pm0.12\times10^7$
2	3.29	1.18	$2.8\pm0.10\times10^8$	5.63	0.45	$7.4\pm0.29\times10^6$	4.09	1.28	$1.9\pm0.05\times10^8$	5.93	0.35	$2.7\pm0.18\times10^7$
3	3.69	1.43	$1.8\pm0.09\times10^8$	4.48	1.15	$4.2\pm0.19\times10^3$	3.88	1.45	$1.2\pm0.04\times10^8$	5.15	0.83	$3.6\pm0.20\times10^6$
4	3.59	1.20	$2.0\pm0.09\times10^6$	4.08	1.13	$1.1\pm0.05\times10^2$	3.66	1.41	$4.3\pm0.19\times10^6$	4.64	0.98	$2.3\pm0.15\times10^5$

All data are expressed as mean±SD from three different experiments (each experiment was conducted in triplicate).

et al., 2008a, c). The extracellular proteases might hydrolyze SPP to provide nutrients for this strain during fermentation. Consequently, hydrolysates of casein and SPP were added to the medium at 2.5% (v/v) as a prebiotic carbon/nitrogen source for *L. paracasei* subsp. *paracasei* TKU010 (Fig. 1). Growth was enhanced in MRS medium when carbon/nitrogen sources of hydrolysates of casein and SPP were added. The hydrolysate of casein enhanced the growth of TKU010 most obviously (17.20–18.25 OD_{660}), followed by the hydrolysate of SPP (16.00–15.06 OD_{660}). Interestingly, the bacterium grew very poorly on casein (2.5%, v/v), in comparison with its growth on hydrolysates. The electrophoresis of proteins from the hydrolysates of casein and SPP resulted in some bands (data not shown), and indicated the presence of smaller proteins with a molecular weight lower than that of α-lactalbumin (14.4 kDa). Thus, it is postulated that peptides present in the hydrolysates are responsible for enhancing the growth of this strain. It is interesting to note that TKU010 was able to grow better on medium with peptides added than on MRS alone. However, it is not possible from the in vitro yield and growth rate data alone to determine which would provide the most selective and effective prebiotic for this organism in vivo. The results strongly indicated that hydrolysates of casein and SPP could be a suitable growth medium for this organism and the fermented SPP product could be further developed into a functional beverage. The conversion of SPP to a valuable product could also reduce economic and environmental liabilities.

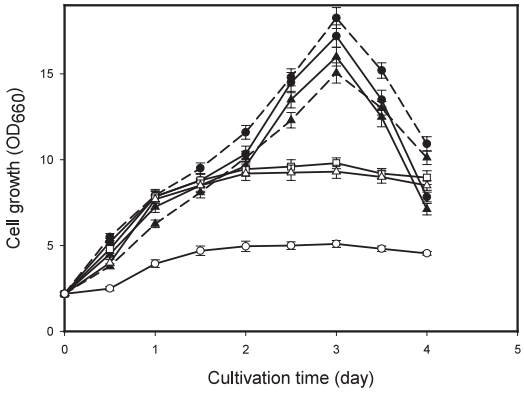

Fig. 1. The effect of carbon/nitrogen source on the cell yield of *L. paracasei* subsp. *paracasei* TKU010.

—▲—, hydrolysate of SPP by bromelain; --▲--, hydrolysate of SPP by *B. subtilis* TKU007 protease; —●—, hydrolysate of casein by bromelain; --●--, hydrolysate of casein by *B. subtilis* TKU007 protease; —△—, SPP; —○—, casein; —□—, MRS alone.

Antimicrobial activity of the culture supernatant

Antibacterial effect. *Lactobacillus* has been extensively applied to food. This means that *Lactobacillus* and its fermentation products are acceptable to humans. It has also been well documented that short peptides produced by enzymatic hydrolysis and fermentation from different sources of protein have immunomodulatory effects, and antimicrobial and antioxidant activity (Gibbs et al., 2004; He et al., 2006; Kristinsson and Rasco, 2000). The culture supernatants of TKU010 and TKU012 were prepared under the optimal culture conditions of extracellular protease production (Wang et al., 2008a, c) and the culture supernatants of *L. paracasei* subsp. *paracasei* BCRC12193 and *L. kefir* BCRC14011 were also prepared with the culture conditions of *L. paracasei* subsp. *paracasei* TKU010. These culture supernatants were all used to investigate the antibacterial effect on *E. coli* BCRC13086. The results showed only TKU010 culture supernatant had antibacterial effect on *E. coli* BCRC13086. However, no growth inhibition on *E. coli* BCRC13086 was found with the culture supernatant of *L. paracasei* subsp. *paracasei* TKU012, *L. paracasei* subsp. *paracasei* BCRC12193 or *L. kefir* BCRC14011. The effect of different culture times (1–4 days) by TKU010 on the antibacterial effect on *E. coli* BCRC13086 was further investigated. It was found that the culture supernatants had antibacterial activity on the first day and the fourth day (Fig. 2). Compared with the optimal protease production conditions, the difference in culture time was that the optimal protease production was on the third day (Fig. 3) but the production of antibacterial materials was on the first day and the fourth day. As for the fourth day culture supernatants of TKU010, the antibacterial activity was found after the production of protease. The results suggested that antibacterial peptides might be hydrolyzed by TKU010 protease and present in the culture supernatant. As for the first day culture supernatants of TKU010, no protease activity was found (Fig. 3). Therefore, the antibacterial materials might be bacteriocins produced by TKU010. Lactic acid bacteria (LAB) are well known for their production of antimicrobial compounds, including antibacterial peptides, collectively known as bacteriocins. Todorov and Dicks (2006) also found that bacteriocin-producing lactic acid bacteria from boza had antibacterial activity.

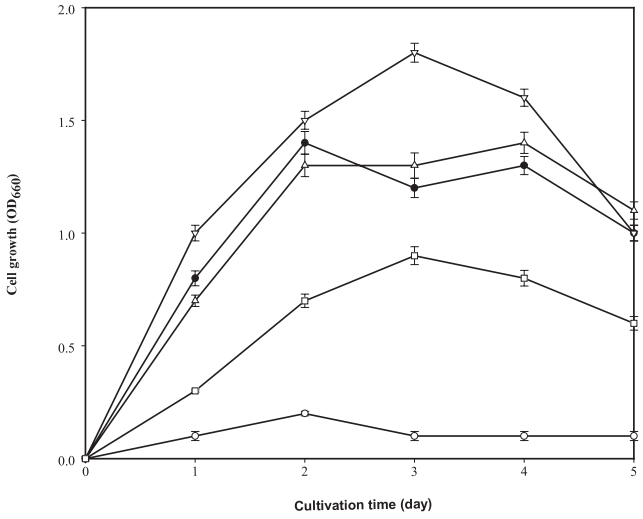

Fig. 2. Growth inhibition on *E. coli* BCRC13086 with the supernatants of different culture times by *L. paracasei* subsp. *paracasei* TKU010.

The supernatants of different culture times were prepared by using squid pen powder (1%) as a sole carbon/nitrogen source and treated in *E. coli* BCRC13086 growth. ●, control; ○, the first day culture supernatant; ▽, the second day culture supernatant; △, the third day culture supernatant; □, the fourth day culture supernatant.

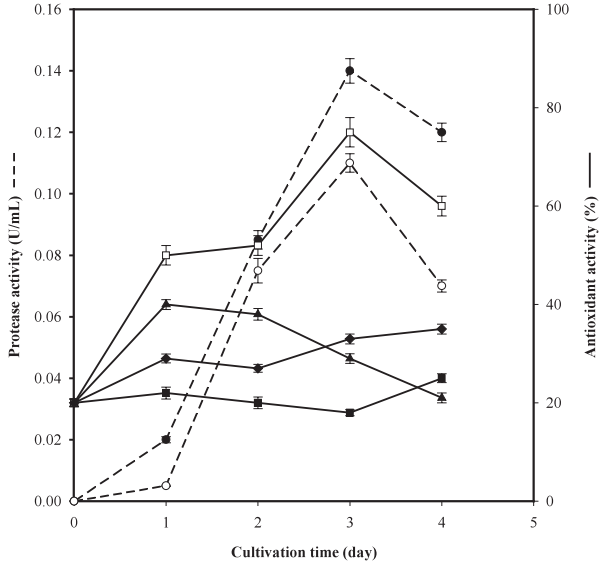

Fig. 3. Time courses of antioxidant and protease activity in the culture of *L. paracasei* subsp. *paracasei* TKU010, *L. paracasei* subsp. *paracasei* TKU012, *L. paracasei* subsp *paracasei* BCRC12193 and *L. kefir* BCRC14011.

TKU010 and TKU012 were incubated under the optimal culture conditions of extracellular protease production and analyzed for antioxidant activity, protease activity of the culture supernatant. --○--, TKU010 protease; --●--, TKU012 protease; —□—, TKU010 antioxidant; —■—, TKU012 antioxidant; —◆—, BCRC12193 antioxidant; —▲—, BCRC14011 antioxidant.

Antifungal effect. The culture supernatants of *L. paracasei* subsp. *paracasei* TKU010, *L. paracasei* subsp. *paracasei* TKU012, *L. paracasei* subsp. *paracasei* BCRC12193 and *L. kefir* BCRC14011 were also used to investigate the antifungal effects. The fungi tested were phyto-pathogenic mold *F. oxysporum* BCRC32121 and human pathogenic mold *A. fumigatus* BCRC30099. Only TKU010 culture supernatants on the first day and the fourth day of incubation had 0.5 U and 0.4 U inhibitive effects on *A. fumigatus* BCRC30099, respectively (data not shown). However, no any antifungal activity was found in the culture supernatants of *L. paracasei* subsp. *paracasei* TKU012, *L. paracasei* subsp. *paracasei* BCRC12193 or *L. kefir* BCRC14011 (data not shown). As for the inhibition on *F. oxysporum* BCRC32121, the inhibitive effect of TKU010 culture supernatants was similar to that of *A. fumigatus* BCRC30099. Similarly it has also been observed that *Bacillus cereus* QQ308 culture supernatant inhibited spore germination and germ tube elongation of *F. oxysporum, F. solani,* and *P. ultimum* (Chang et al., 2007). About the antifungal materials, besides bacteriocins, it is conjectured that antifungal peptides might be hydrolyzed by TKU010 protease and present in the culture supernatant. To our knowledge, the mechanism by which an antimicrobial peptide executes its function depends on a number of physicochemical properties: the amino acid sequence, net charge, amphipathicity, hydrophobicity, structural folding in membranes, oligomerization, peptide concentration, and membrane composition. There is an urgent need for exploration of the mechanisms by which antimicrobial factors inhibit the growth of potentially pathogenic fungi. Currently investigations are being performed to achieve the above. Although much work remains to be done before its application in the field, the results of antifungal effects to date suggest that the development of TKU010 as a probiotic bacterium in functional foods or a biocontrol agent is an environmentally benign alternative to current disease control strategies.

Antioxidant property of the culture supernatant

Strain TKU010 and TKU012 are protease-producing lactobacilli, and the purification of the extracellular proteases by TKU010 and TKU012 using squid pen as carbon/nitrogen source had been reported (Wang et al., 2008a, c). Besides, it has been reported that peptides have antioxidative (He et al., 2006; Lin and Chou, 2004; Pinero Estrada et al., 2001; Xing et al., 2005)

and anticarcinogenic (Liang et al., 2007; Wang et al., 2008b) properties. To increase the utilization of the chitin/protein-containing marine wastes, we incubated TKU010 and TKU012 for 1−4 days with squid pen powder under the optimal culture conditions of extracellular protease production (Wang et al., 2008a, b) and analyzed the antioxidant activity and protease activity of the culture supernatant. *L. paracasei* subsp. *paracasei* BCRC12193 and *L. kefir* BCRC14011 were used as the reference strains with the culture conditions of *L. paracasei* subsp. *paracasei* TKU010 when the antioxidant activity was analyzed. The antioxidant activity assayed was the scavenging ability on DPPH. After heating squid pen wastes in an autoclave (121˚C for 15 min), antioxidant activity (about 20% per ml) was found in the supernatants. However, as shown in Fig. 3, it was found that the antioxidant activity increased significantly after fermenting by TKU010, and increased slightly after fermenting by *L. paracasei* subsp. *paracasei* BCRC12193 or *L. kefir* BCRC14011. However, no antioxidant activity was found in TKU012 culture supernatants (Fig. 3). TKU010 culture supernatant (1% SPP) incubated for 3 days had the highest protease activity and antioxidant activity; the DPPH scavenging ability of TKU010 culture supernatant was raised about 75% per ml (Fig. 3). The results supposed that antioxidant peptides might be hydrolyzed by TKU010 protease and present in the culture supernatant. The antioxidant materials may contain peptides that are electron donors and can react with free radicals to terminate the radical chain reaction. TKU010 culture supernatant displayed much more antioxidant activity than the supernatant after heating in an autoclave (121˚C for 15 min). It is assumed that even though the treatment (121˚C for 15 min) degrades squid pen waste and produces some of the antioxidant materials, most of the antioxidant materials are produced by strain TKU010.

Both TKU010 serine protease and TKU012 metalloprotease were produced by the strains of the same species *L. paracasei* subsp. *paracasei* and had similar molecular weights (49 kDa) (Wang et al., 2008a, c). However, the culture supernatant of squid pen powder fermented by these two lactobacilli strains showed obvious difference in antimicrobial and antioxidant activity. Consequently, peptide mass mapping was used to compare the sequence difference between TKU010 protease and TKU012 protease. Peptide mass mapping was performed by Mission Biotech Co. (Taipei, Taiwan) using LC/MS as described in a previous paper (Wang et al., 2008a, c). Peptide masses obtained were searched against a comprehensive nonredundant protein sequence database (NCBINr) using the Mascot program for protein identification. From the comparison between the peptide mass mappings of TKU010 and TKU012 protease, it was shown that these two proteases had 7 similar peptides. TKU010 protease had two more peptides, than TKU012, AATTGYDAVDDLLHYHER and QTFTHEIGHA LGLSHPGDYNAGEGNPTYR. Among them, the peptide AATTGYDAVDDLLHYHER is the N-terminal initial sequence of *Serratia* protease (serralysin). These structural differences between TKU010 protease and TKU012 protease might be the reason for the above different properties including the effect of antimicrobial and antioxidant activity.

Conclusion

In the present study, we report the characterization of a novel strain *Lactobacillus paracasei* subsp. *paracasei* TKU010, and its application in antimicrobial and antioxidant activity by conversion of squid pen. TKU010 was more resistant to the low pH of the stomach, human gastric juice, bile, and lysozyme than TKU012 and YQ101. The hydrolysate of casein could enhance the growth of TKU010 most obviously, followed by the hydrolysate of SPP. In addition, incubating with SPP, the culture supernatant of TKU010 also showed antimicrobial and antioxidant physiological activities, and revealed its hidden potential in the production of biocontrol agents and functional foods. Further studies should be done concerning the performance of TKU010 culture supernatant in vivo.

Acknowledgments

This work was supported in part by a grant of the National Science Council, Taiwan (NSC96-2313-B-032-002-MY3).

References

Brashears, M. M., Jaroni, D., and Trimble, J. (2003) Isolation, selection, and characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157:H7 in cattle. *J. Food Prot*., **66**, 355−363.

Chang, J. H., Chou, C. C., and Li, C. F. (2000) Growth and survival of *Escherichia coli* O157:H7 during the fermentation

and storage of diluted cultured milk drink. *Food Microbiol.*, **17**, 579–587.

Chang, W. T., Chen, Y. C., and Jao, C. L. (2007) Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. *Biores. Technol.*, **98**, 1224–1230.

Chou, L. S. and Weimer, B. (1999) Isolation and characterization of acid and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *J. Dairy Sci.*, **82**, 23–31.

Conway, P. L., Gorbach, S. L., and Goldin, B. R. (1987) Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J. Dairy Sci.*, **70**, 1–12.

Gibbs, B. F., Zougman, A., Masse, R., and Mulligan, C. (2004) Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Res. Int*., **37**, 123–131.

Gilliland, S. E., Stanley, T. E., and Bush, L. J. (1984) Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *J. Dairy Sci.*, **67**, 3045–3051.

Gilliland, S. E. and Walker, D. K. (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J. Dairy Sci*., **73**, 905–911.

Gupta, P. K., Mital, B. K., and Garg, S. K. (1996) Characterization of *Lactobacillus acidophilus* strains for use as dietary adjunct. *Int. J. Food Microbiol.*, **29**, 105–109.

He, H., Chen, X., Sun, C., Zhang, Y., and Gao, P. (2006) Preparation and functional evaluation of oligopeptide-enriched hydrolysate from shrimp (*Acetes chinensis*) treated with crude protease from *Bacillus* sp. SM98011. *Biores. Technol*., **97**, 385–390.

Hyronimus, B., Le Marrec, C., Hadj Sassi, A., and Deschamps, A. (2000) Acid and bile tolerance of spore-forming lactic acid bacteria. *Int. J. Food Microbiol.*, **61**, 193–197.

Kristinsson, H. G. and Rasco, B. A. (2000) Biochemical and functional various alkaline proteases. *J. Agric. Food Chem*., **48**, 657–666.

Kyung, Y. Y., Woodams, E. E., and Hang, Y. D. (2005) Fermentation of beet juice by beneficial lactic acid bacteria. *LWT-Food Sci. Technol.*, **38**, 73–75.

Liang, T. W., Chen, Y. J., Yen, Y. H., and Wang, S. L. (2007) The antitumor activity of the hydrolysates of chitinous materials hydrolyzed by crude enzyme from *Bacillus amyloliquefaciens* V656. *Process Biochem*., **42**, 527–534.

Lin, H. Y. and Chou, C. C. (2004) Antioxidant activities of water-soluble disaccharide chitosan derivatives. *Food Res. Int.*, **37**, 883–889.

Lin, W. H., Hwang, C. F., Chen, L. W., and Tsen, H. Y. (2006) Viable counts, characteristic evaluation for commercial lactic acid bacteria products. *Food Microbiol.*, **23**, 74–81.

Noh, D. O. and Gilliland, S. E. (1993) Influence of bile on cellular integrity and β-galactosidase activity of *Lactobacillus acidophilus*. *J. Dairy Sci.*, **76**, 1253–1259.

Ong, L., Henriksson, A., and Shah, N. P. (2007) Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus*, *Lb. paracasei*, *Lb. casei* or *Bifidobacterium* sp. *Int. Dairy J.*, **17**, 67–78.

Pinero Estrada, J. E., Bermejo Bescós, P., and Villar del Fresno, A. M. (2001) Antioxidant activity of different fractions of *Spirulina platensis* protein extract. *Farmaco*, **56**, 497–500.

Shimada, K., Fujikawa, K., Yahara, K., and Nakamura, T. (1992) Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.*, **40**, 945–948.

Suscovic, J., Brkic, B., Matosic, S., and Maric, V. (1997) *Lactobacillus acidophilus* M92 as potential probiotic strain. *Milchwissenschaften*, **52**, 430–435.

Todorov, S. D. and Dicks, L. M. T. (2006) Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria. Comparison of the bacteriocins. *Process Biochem.*, **41**, 11–19.

Wang, S. L., Huang, T. Y., Wang, C. Y., Liang, T. W., Yen, Y. H., and Sakata, Y. (2008a) Bioconversion of squid pen by *Lactobacillus paracasei* subsp. *paracasei* TKU010 for the production of proteases and lettuce growth enhancing biofertilizers. *Biores. Technol.*, **99**, 5436–5443.

Wang, S. L., Kao, D. Y., Wang, C. L., Yen, Y. H., Chern, M. K., and Chen, Y. H. (2006) A solvent stable metalloprotease produced by *Bacillus* sp. TKU004 and its application in the deproteinization of squid pen for beta-chitin preparation. *Enzyme Microb. Technol.*, **39**, 724–731.

Wang, S. L., Lin, H. T., Liang, T. W., Chen, Y. J., Yen, Y. H., and Guo, S. P. (2008b) Reclamation of chitinous materials by bromelain for the preparation of antitumor and antifungal materials. *Biores. Technol.*, **99**, 4386–4393.

Wang, S. L., Shih, I. L., Liang, T. W., and Wang, C. H. (2002) Purification and characterization of two antifungal chitinases extracellularly produced by *Bacillus amyloliquefaciens* V656 in a shrimp and crab shell powder medium. *J. Agric. Food Chem.*, **50**, 2241–2248.

Wang, S. L., Wang, C. Y., and Huang, C. Y. (2008c) Microbial reclamation of squid pen for the production of a novel extracellular serine protease by *Lactobacillus paracasei* subsp. *paracasei* TKU012. *Biores. Technol.*, **99**, 3411–3417.

Wang, S. L. and Yeh, P. Y. (2006) Production of a surfactant- and solvent-stable alkaliphilic protease by bioconversion of shrimp shell wastes fermented by *Bacillus subtilis* TKU007. *Process Biochem*., **41**, 1545–1552.

Xanthopoulos, V., Litopoulou-Tzanetaki, E., and Tzanetakis, N. (2000) Characterization of *Lactobacillus* isolates frominfant faeces as dietary adjuncts. *Food Microbiol.*, **17**, 205–215.

Xing, R., Yu, H., Liu, S., Zhang, W., Zhang, Q., and Li, Z. (2005) Antioxidative activity of differently regioselective chitosan sulfates in vitro. *Bioorg. Med. Chem.*, **13**, 1387–1392.