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Influence of Medium Components and Growth Conditions on Pediocin Iz3.13 Production by *Pediococcus pentosaceus* Iz3.13, Isolated from Japanese Traditional Fermented Seafood

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Abstract

Growth requirements for maximum production of pediocin Iz3.13 by *Pediococcus pentosaceus* Iz3.13 were studied. Physical and chemical parameters that enhanced pediocin production included incubation in static broth at 30°C, with an initial pH of 4.5. Optimum nutritional conditions that enhanced pediocin Iz3.13 production were 3.5% Trypticase peptone, 3.5% Phytone peptone or 5.0% Proteose peptone No. 3 as nitrogen sources, and glucose or maltose as carbon sources. Supplementing growth media with beef extract and $MgCl_2$ also enhanced production. This study proposes an optimized growth medium containing 5.0% Proteose peptone No. 3, 3.5% Phytone peptone, 2.5% glucose, 1.5% $MgCl_2$, 3.0% beef extract and 0.25% K_2HPO_4. This medium significantly enhanced pediocin Iz3.13 production (5.1 fold increase) as compared to a basal medium.

Key words: Bacteriocin, Pediocin, *Pediococcus*, Medium components, Culture condition, Optimization

Introduction

Bacteriocins are ribosomally synthesized antibacterial peptides or proteins, produced by many types of bacteria. Lactic acid bacteria (LAB) produce bacteriocins that can inhibit food-borne pathogens, such as *Listeria monocytogenes*, *Clostridium botulinum*, and spoilage bacteria, like *Bacillus coagulans* and *Alicyclobacillus acidoterrestris*, in food (Yamazaki et al., 2000; Azuma et al., 2007; Galvez et al., 2007).

Bacteriocin production is strongly dependent on factors like carbon, nitrogen sources and fermentation conditions such as medium pH, incubation temperature and agitation (Biswas et al., 1991; Abriouel et al., 2001; Nel et al., 2001; Delgado et al., 2005; Todorov et al., 2006; Somkuti and Gilbreth, 2007). Moreover a recent review of available literature indicates that optimum conditions for bacteriocin production are strain dependent (Papagianni and Anastasiadou, 2009). For a bacteriocin or its producing strain to be practical use in food safety, optimum conditions for bacteriocin production must be understood well.

Pediococcus pentosaceus Iz3.13 was recently isolated from Japanese traditional fermented seafood (Bagenda et al., 2008). The strain produced a pediocin-like bacteriocin and is the first *Clostridium botulinum*-inhibiting bacteriocin producer.

In this paper, we report the effects of nutrients, medium pH and cultivation conditions on the production of pediocin by *P. pentosaceus* Iz3.13.

Materials and Methods

Bacteriocin producing strain and growth conditions

P. pentosaceus isolated from fermented Japanese food (Bagenda et al., 2008) was grown in Tryptic soy broth (Difco, Sparks, MD, U.S.A.) supplemented with 0.5% yeast extract (Difco) (TSBYE) at 30°C.

Bacteriocin assay

Bacteriocin activity was assayed using the well diffusion method, as described previously (Yamazaki et al., 2003). In brief, *Listeria monocytogenes* IID 580 was grown in TSBYE at 30°C for 12 h, and inoculated into molten Tryptic soy agar (Difco) supplemented with 0.5% yeast extract (TSAYE, Difco) (initial inoculum 10^6 CFU/ml). The inoculated agar medium (20 ml) was pipetted into sterile petri dishes. After solidification, 6

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mm wells were made with a cork borer. Fifty microliters of serial 1.5-folds dilutions of supernatant were placed into the wells. The plates were incubated at 4°C for 12 h and then at 30°C for 12 h. The bacteriocin titer was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strain and was expressed as arbitrary units per milliliter (AU/ml).

Effect of medium pH and agitation on pediocin Iz3.13 production

A complex medium containing 1.7% (w/v) Trypticase peptone (Difco), 0.3% Phytone peptone (Difco), 0.25% glucose (Kanto chemical. Co. ltd., Tokyo, Japan), 0.5% NaCl (Wako chemical. Co. ltd., Osaka, Japan), 0.5% yeast extract and 0.25% K_2HPO_4 (Kanto) was used in this study. Since *P. pentosaceus* Iz 3.13 grew well in TSBYE (pH 7.0), it was used as a basal medium. Flasks containing the basal medium, whose pH was adjusted to 4.5, 5.0 and 6.0 by 1 M HCl and 7.0 by 1 M NaOH, were autoclaved. An overnight culture of *P. pentosaceus* Iz3.13 was inoculated (1.0% v/v) into each medium and incubated at 30°C with or without agitation (40 r.p.m., Bio-Photorecoder TVS062CA, Advantec, Tokyo, Japan). Bacteriocin activity, pH and cell growth ($O.D._{660}$) were determined after 24, 36 and 48 h incubation using the well diffusion method, a compact pH meter (Horiba B-212, Kyoto, Japan) and a UV/VIS spectrophotometer (JASCO V-530, Tokyo, Japan), respectively.

Effect of nitrogen sources on pediocin Iz3.13 production

Trypticase peptone and Phytone peptone in the basal media were replaced with other nitrogen sources and the effect of this replacement on bacteriocin production was studied. Alternative nitrogen sources tested were; Polypeptone peptone (BBL, Sparks, MD, U.S.A.), Thiotone E peptone (Difco), Proteose peptone (Difco), Proteose peptone No. 3 (Difco), Casamino acid (Difco), Polypeptone-Y (Nihon Seiyaku, Tokyo, Japan), Peptone aus fleish (peptone from meat peptic digested, Merck, Darmtstadt, Germany) and Peptone aus gelatin (peptone from gelatin pancreatic digested, Merck). Concentrations of alternative nitrogen sources were 1.5, 2.5, 3.5 and 5.0% (w/v). Each of the media was then adjusted to pH 4.5 with 1 M HCl, autoclaved, and inoculated with an overnight culture of *P. pentosaceus* Iz3.13 (1.0% v/v). Inoculated media were incubated at 30°C for 48 h without agitation. Bacteriocin activity, medium pH and cell growth ($O.D._{660}$) of culture supernatants were measured as described above. When the effect of Phytone peptone (Difco) was studied, a basal medium without Phytone peptone was used.

Effect of carbon sources on pediocin Iz3.13 production

The effect of carbon sources on pediocin production was investigated by adjusting the concentration of glucose or substituting it with alternative carbon sources at varying concentrations. Glucose in the basal medium was substituted for with arabinose (Wako), cellobiose (Wako), fructose (Wako), galactose (Wako), maltose (Wako), mannose (Wako), starch (Wako), sucrose (Kanto), trehalose (Wako), raffinose (Wako), xylose (Wako), or *N*-acetyl glucosamine (Wako) at concentrations of 1.0, 2.0, 2.5, 3.0 and 4.0% (w/v). Media were inoculated with *P. pentosaceus* Iz3.13 (1.0% v/v) and incubated at 30°C for 48 h without agitation. Bacteriocin activity, medium pH and cell growth ($O.D._{660}$) were measured.

Effect of inorganic salts on pediocin Iz3.13 production

The effect of inorganic salts on pediocin production was investigated using a basal medium in which NaCl was substituted for with other inorganic salts at various concentrations. KCl (Kanto), $MgCl_2 \cdot 6H_2O$ (Wako), $CaCl_2 \cdot 2H_2O$ (Wako), $MgSO_4 \cdot 7H_2O$ (Kanto) or Na_2SO_4 (Wako) were added at final concentrations of 1.0, 1.5, 2.5, 3.0 and 5.0% (w/v) to a basal medium lacking NaCl. The adjusted media were inoculated with *P. pentosaceus* Iz3.13 (1.0% v/v) and incubated at 30°C for 48 h without agitation.

Effect of vitamin sources on pediocin Iz3.13 production

The effect of substituting yeast extract in the basal medium with alternative vitamin sources was investigated. Alternative vitamin sources used in this study included beef extract (Difco), malt extract (Difco), bonito extract (Wako), Lab-lemco powder (Oxoid, Basingstoke, Hampshire, England) or liver powder (Difco). Concentrations of the alternative vitamin sources were 1.5, 2.0, 2.5, 3.0 and 5.0% (w/v). Adjusted media were inoculated with *P. pentosaceus* Iz3.13 (1.0% v/v) and incubated at 30°C for 48 h without agitation.

Evaluation of pediocin Iz3.13 production in optimized media

Based on the results from the above experiments, two optimized media, A and B, were developed and pediocin Iz3.13 production in these media was evaluated. Medium A contained 3.5% Trypticase peptone, 3.5% Phytone peptone, 2.5% glucose, 1.5% $MgCl_2$, 3.0% beef extract and 0.25% K_2HPO_4. Medium B contained 3.5% Proteose peptone No. 3, 3.5% Phytone peptone, 2.5% glucose, 1.5% $MgCl_2$, 3.0% beef extract and 0.25%

K_2HPO_4. Both media were adjusted to pH 4.5 with 1 N HCl before autoclaving. Media were inoculated with *P. pentosaceus* Iz3.13 (1.0% v/v) and incubated at 30°C without agitation. Bacteriocin activity, medium pH and cell growth were monitored at 12-hour intervals.

Results and Discussion

Effect of culture conditions on pediocin Iz3.13 production

It has been reported that during culturing of bacteriocin producing strains in broth media, bacteriocin activity reaches a maximum at the end of the exponential growth phase, and then decreases rapidly after the maximum cell density (10^8 to 10^9 CFU/ml) has been reached (Yamazaki et al., 2003). Chinachoti et al. (1997) suggested that aeration reduced bacteriocin production due to higher cell growth and therefore higher production of degrading enzymes. In the case of pediocin Iz3.13, activity did not decrease rapidly after reaching maximum cell density had been achieved (Bagenda et al., 2008). This indicates that the producer strain, *P. pentosaceus* Iz3.13 does not produce extracellular enzymes to degrade pediocin Iz3.13. For purposes of this study, it considered sufficient to measure bacteriocin activity during the stationary phase (after 48 h of incubation at 30°C).

Pediocin Iz3.13 production in static TSBYE media was higher than that in agitated TSBYE media for all tested pH values. However, growth of *P. pentosaceus* Iz3.13 in static TSBYE media was slower than that in agitated media. Under static conditions, pediocin Iz3.13 activity was 52.5, 118.2, 118.2 and 227.8 AU/ml at pH 7.0, 6.0, 5.0 and 4.5, respectively. On the contrary, when the media were agitated, pediocin Iz3.13 activity was 30.0, 40.0, 52.5 and 67.5 AU/ml at pH 7.0, 6.0, 5.0 and 4.5, respectively (Table 1). Stress factors like low temperature, low pH and high osmolarity have been shown to enhance bacteriocin production (De Vuyst et al., 1996 ; Aasen et al., 2000 ; Sashihara et al., 2001). De Vuyst et al. (1996) suggested that slow growth enhanced bacteriocin production, because more energy was available for bacteriocin biosynthesis. Actually, growth of strain Iz3.13 was slower in static cultures than in agitated cultures. The results reported here emphasize the importance of stress factors and energy conservation in enhancing pediocin production.

Table 1. Effect of initial pH and physical parameters on pediocin Iz3.13 production

Motion status	Initial pH	OD_{660nm}		Final pH	Maximum activity (AU/ml)
		24 h	48 h		
Agitated	7.0	0.898	0.959	5.4	30.0
	6.0	0.850	0.953	4.5	40.0
	5.0	0.705	0.844	4.1	52.5
	4.5	0.639	0.745	4.0	67.5
Static	7.0	0.232	0.710	5.2	52.5
	6.0	0.321	1.001	4.5	118.2
	5.0	0.125	0.920	4.2	118.2
	4.5	0.362	0.786	3.9	227.8

The effect of initial pH of medium was also evaluated in static and agitated TSBYE media. In both cases, the bacteriocin activity increased at low initial pH, but not when the initial pH was neutral. Moreover, pediocin Iz3.13 production at pH 4.5 was four-fold higher (227.8 AU/ml) than that achieved in static TSBYE with an initial pH 7.0. Further, pediocin production in static TSBYE with initial pH 4.5 was 7.6-fold higher than that in non-static TSBYE with initial pH 7.0. These results were expected since it is known that pediocin production is best below pH 4.5 and minimal above pH 5.0 (Biswas et al., 1991 ; Yang et al., 1994 ; Todorov and Gilbreth, 2009). Pediocin production at low pH is attributed to post-translational processing of the bacteriocin (Biswas et al., 1991 ; Verellen et al., 1998). From these results, optimum physical parameters for highest pediocin Iz3.13 production were static incubation at 30°C with an initial pH of 4.5.

Effect of medium components on pediocin Iz3.13 production

Effect of various nitrogen sources on pediocin Iz3.13 production is shown Fig. 1. In the presence of 3.5% Trypticase peptone or 5.0% Proteose peptone No. 3 as the main nitrogen source, pediocin Iz3.13 activity (512.6 AU/ml) was 2.3-fold higher than that in the basal medium (TSBYE, pH 4.5). Compared to the basal medium, Phytone peptone enhanced pediocin Iz3.13 production by at least 300 AU/ml at all concentrations. Maximum activity was less with Proteose peptone, Peptone aus fleish, Peptone aus gelatin, Polypeptone Y and Casamino acids. Similar findings have been reported elsewhere. De Carvalho et al. (2009) reported that bovicin HC5 production was enhanced by Trypticase peptone or meat peptone, and a combination of Trypticase peptone and yeast extract. Kim et al. (2006) and Todorov and Dicks (2006) also reported that Tryptone was the best nitrogen source for micrococcin GO5 production and a bacteriocin produced by *Lactobacillus plantarum* ST341LD, respectively. From the findings of the present study it was suggested that the most suitable nitrogen sources for pediocin Iz3.13 production are 3.5% Trypticase peptone or 5.0% Proteose peptone No. 3, followed by 3.5% Phytone peptone.

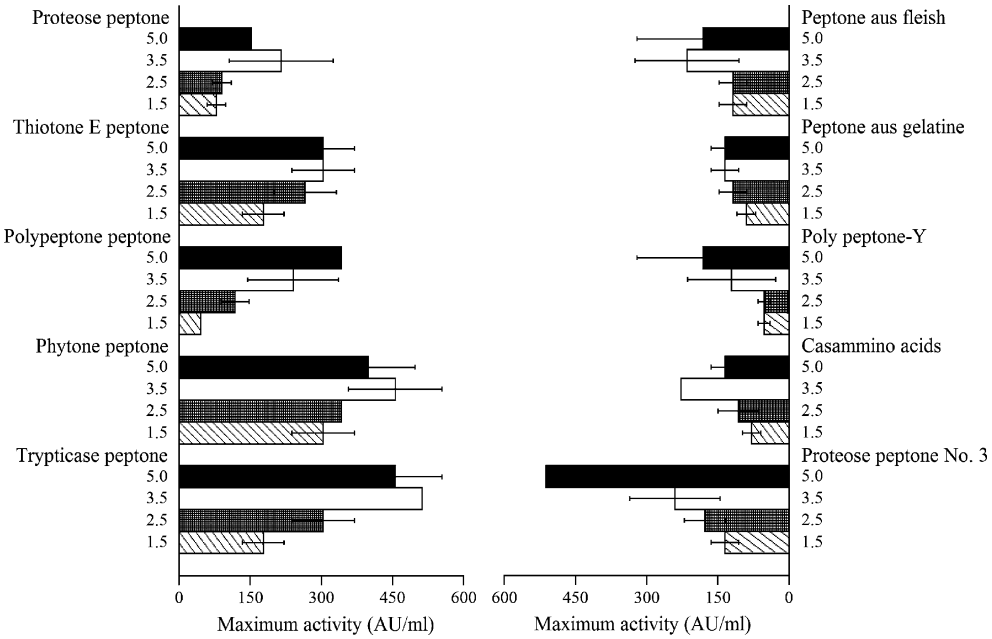

Fig. 1. Effect of nitrogen sources on the production of pediocin Iz3.13. *P. pentosaceus* Iz3.13 was inoculated into basal media made with different nitrogen sources and incubated at 30°C for 48 h. Pediocin Iz3.13 activity in the culture supernatants was determined by the well diffusion assay using *L. monocytogenes* IID580 as the indicator organism. The data represent the means and standard deviations of three independent experiments.

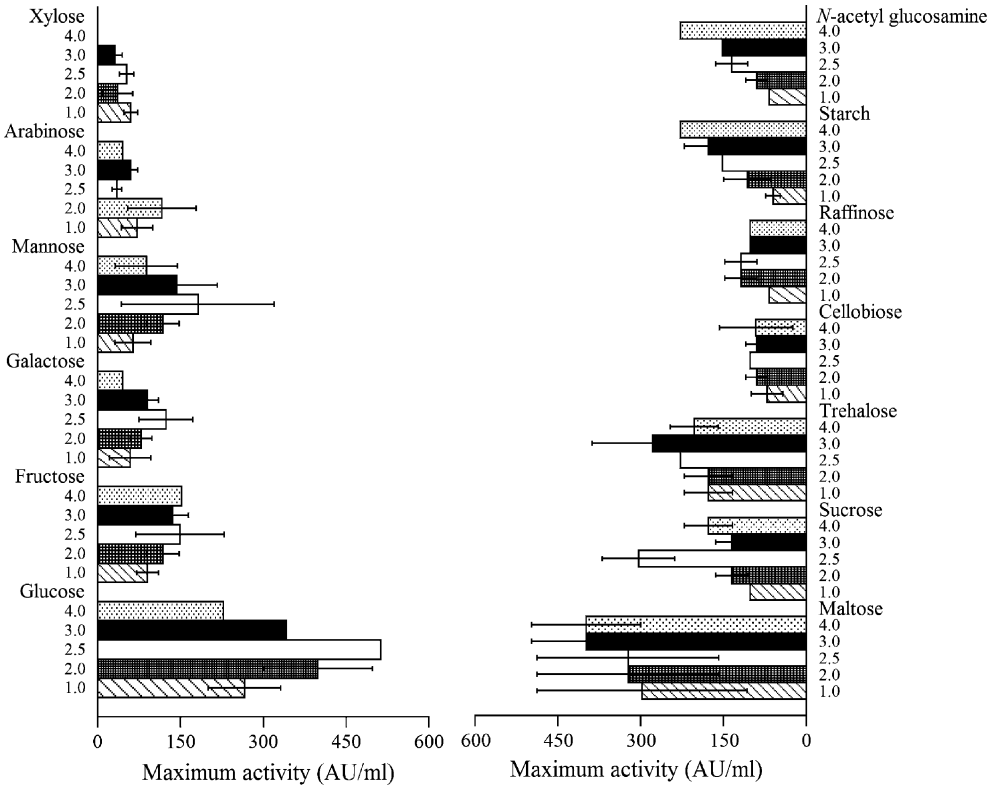

Fig. 2. Effect of carbon sources on the production of pediocin Iz3.13. Various carbon sources at different concentrations (1–4%) were used to substitute glucose. Pediocin Iz3.13 activity in the culture supernatants was determined by the well diffusion assay using *L. monocytogenes* IID580 as the indicator organism. *P. pentosaceus* Iz3.13 cultures were incubated at 30°C for 48 h. The data represent the means and standard deviations of three independent experiments.

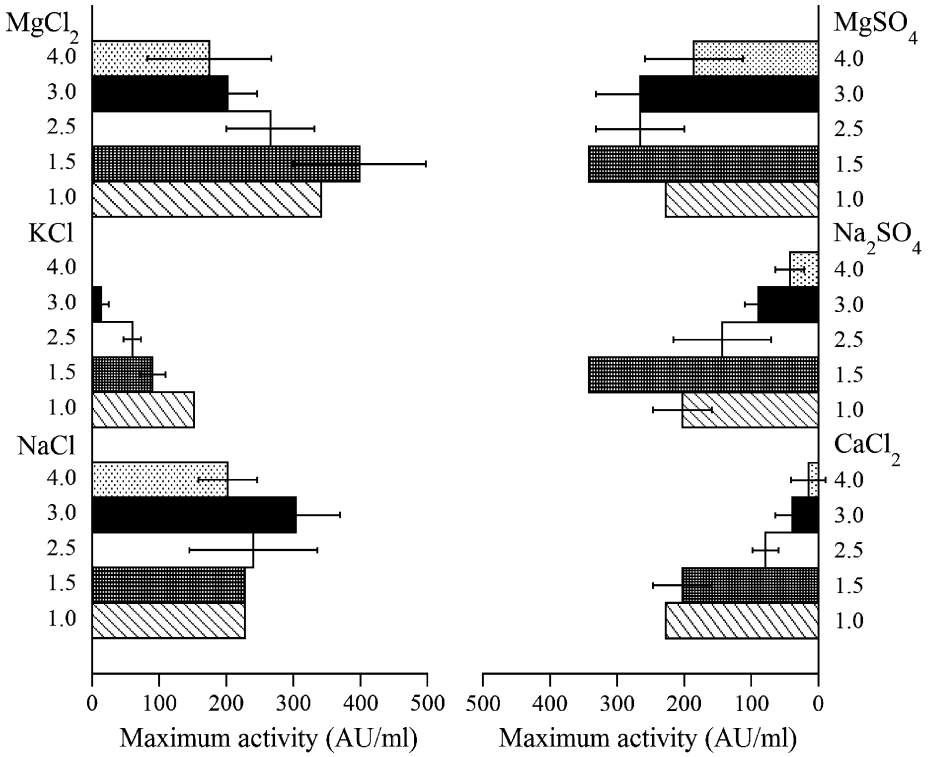

Fig. 3. Effect of inorganic salts on the production of pediocin Iz3.13. Basal media in which NaCl was substituted for with various inorganic salts at different concentrations were used. Pediocin Iz3.13 activity in the culture supernatants was determined by the well diffusion assay using *L. monocytogenes* IID580 as the indicator organism. *P. pentosaceus* Iz3.13 cultures were incubated at 30°C for 48 h. The data represent the means and standard deviations of three independent experiments.

Effect of carbon source on pediocin Iz3.13 production is shown Fig. 2. Maximum activity was generally higher in the presence of glucose or maltose. The highest maximum activity (512.6 AU/ml) was observed when the carbon source was 2.5% glucose. This maximum activity was 2.3-folds higher than that in the basal medium (TSBYE, pH 4.5). Lower maximum activities were observed when the carbon sources were xylose, arabinose, galactose, raffinose, cellobiose or galactose. These results agree with those from previous studies. Chen et al. (2007) reported that fructo-oligosaccharides and trehalose were good carbon sources for bacteriocin production, while raffinose was not. It has also been reported that glucose is a better carbon source for bacteriocin production than cellobiose (De Carvalho et al., 2009). Considering optimal glucose concentration for bacteriocin production, De Vuyst et al. (1996) suggested 2%. Futhermore, Parennte et al. (1997) and Aasen et al. (2000) reported that the addition of more than 2.5% and 4.0% respectively, reduced bacteriocin production. In this study, maximum activity decreased at the glucose concentrations above 3%. Though requirements may vary between strains, it is suggested that the optimum concentration of carbon sources for maximum bacteriocin production is 2.0～4.0%. In the case of pediocin Iz3.13 production the optimum carbon source is 2.5% glucose.

The effect of inorganic salts on pediocin Iz3.13 production is in shown Fig. 3. The highest maximum activity (398.7 AU/ml) was observed in the presence of 1.5% $MgCl_2$. This activity was 1.8-folds higher than that in the basal medium. Furthermore, relatively high maximum activities were observed in the presence of 1.0% $MgCl_2$, 1.5% $MgSO_4$, and 1.5% Na_2SO_4. Thus, higher maximum activities were observed in the presence of magnesium ions. On the other hand, addition of KCl and $CaCl_2$ resulted in relatively low maximum activities. It has been reported that bacteriocin production is often stimulated by inorganic salts as well as pH and temperature. Matsusaki et al. (1996) reported nisin Z produced by *Lactococcus lactis* IO-1 was enhanced by the addition of 0.1 M $CaCl_2$. This was postulated to be due to Ca^{2+} stimulated synthesis processes such as the activation of appropriate prepeptide maturation enzymes and subsequent transport out of the cell. Sashihara et al. (2001) reported that the addition of 1.4 M NaCl stimulated nukacin ISK-1 production, probably activated at the transcriptional level on the nukacin ISK-1 structural gene (*nukA*) by osmotic stress. Uguen et al. (1999) also obtained similar results with Sashihara et al.

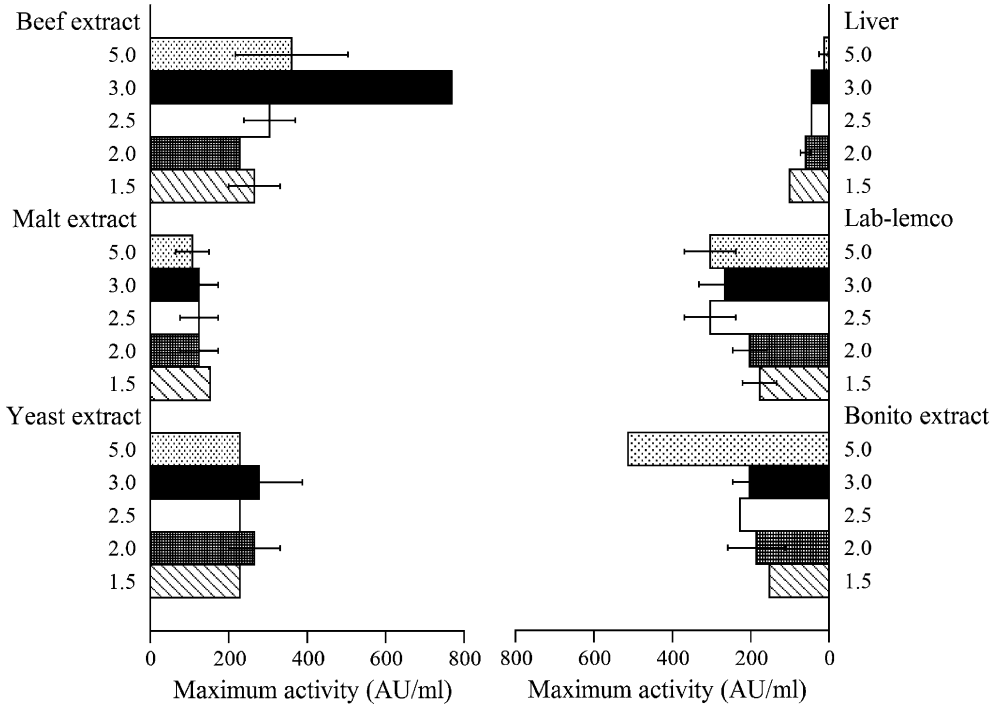

Fig. 4. Effect of vitamin sources on the production of pediocin Iz3.13. Yeast extract in the basal medium was substituted for with various vitamin sources at different concentrations (1.5–5%). Pediocin Iz3.13 activity in the culture supernatants was determined by the well diffusion assay using *L. monocytogenes* IID580 as the indicator organism. *P. pentosaceus* Iz3.13 cultures were incubated at 30°C for 48 h. The data represent the means and standard deviations of three independent experiments.

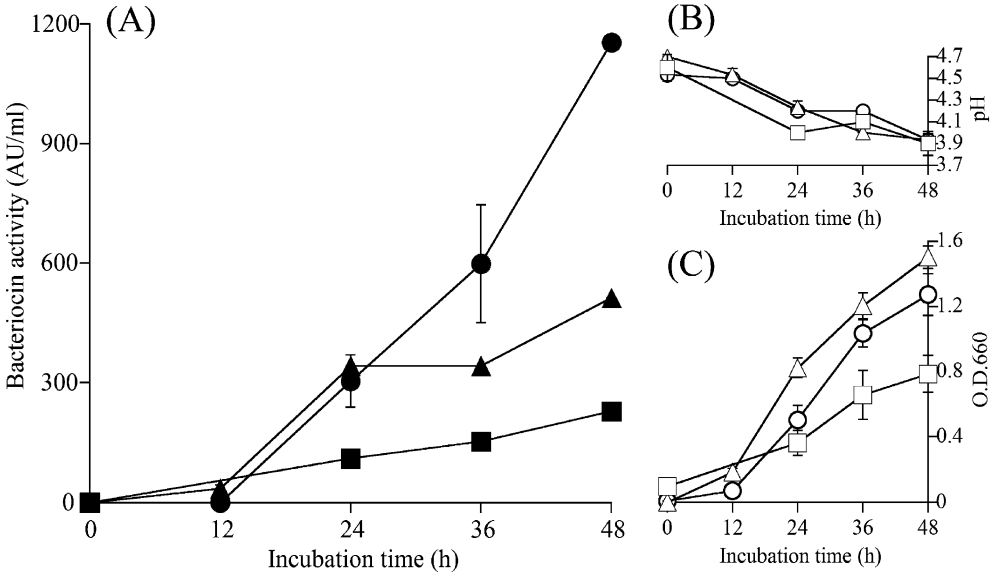

Fig. 5. Pediocin activity (A), medium pH (B), and growth (C) of *P. pentosaceus* Iz3.13 cultured optimized media A (triangles), B (circles) or basal medium (squares). Incubation conditions were 30°C for 48 h. Pediocin Iz3. 13 activity in the culture supernatants was determined by the well diffusion assay using *L. monocytogenes* IID580 as the indicator organism. The data represent the means and standard deviations of three independent experiments.

(2001). It is also known that bacteriocin, once produced, may be adsorbed to the producer cells at neutral pH and released in the presence of salt, acidification and surfactants (Bhunia et al., 1991 ; Todorov et al., 2006). For example, 80% of produced pediocin AcH is reportedly adsorbed to the producer cells at pH 3.5 (Yang et al., 1992). The surface charge of bacterial cells is generally negative (Ibrahim et al., 2001), while that of

bacteriocin molecules and metal ions shows positive. The presence of metal ions could therefore reduce bacteriocin adsorption resulting in an increase in measurable maximum activity. In this study, maximum activity depended on concentration as well as species of the inorganic salt. Maximum activity in the supernatant increased with concentration till the optimum until concentration of the inorganic salt as less bacteriocin was adsorbed to the producer cells. Above the optimum concentration, the excess inorganic salts probably began to interfere with cell growth resulting in a decline in bacteriocin production. From the results, the suitable inorganic salt and optimum concentration for pediocin Iz3.13 production was 1.5% $MgCl_2$.

The effect of various vitamin sources, such as beef extract and yeast extract, on pediocin Iz3.13 production is shown Fig. 4. In the presence of 3.0% beef extract, the highest maximum activity (768.9 AU/ml or 3.4-fold higher than maximum activity in the basal medium) was observed. Bonito extract (5%) also improved pediocin Iz3.13 production. On the contrary, maximum activity was relatively low when the vitamin source was liver powder or malt extract. Cheigh et al. (2002) has been reported that yeast extract is preferable for production bacteriocin from *Lactococcus lactis* subsp. *lactis* A164, while Nel et al. (2001) reported beef extract was more effective for bacteriocin production than yeast extract. Elsewhere, it has been reported that yeast extract was preferable to beef extract (Todorov and Dicks, 2004; Kim et al., 2006; Settanni et al., 2008; De Carvalho et al., 2009). These seemingly differing reports serve to emphasize the strain-dependence of optimal conditions for bacteriocin production. In this study, pediocin Iz3.13 production by *P. pentosaceus* was enhanced by beef extract and bonito extract, but not yeast extract. For higher production of pediocin Iz3.13, the preferred vitamin source and concentration is 3.0% beef extract.

In the final experiment, an effort was made to develop a complex medium based on individual optimal conditions generated in the earlier experiments. Two optimized complex media (medium A and B) were developed and compared (Fig. 5). Pediocin Iz3.13 production in medium B was significantly higher than that in medium A ($p<0.05$), although the growth of the producer cells in medium B was less than that in medium A. Surprisingly, pediocin Iz3.13 maximum activity in medium B was 1,153.3 AU/ml after 48 h incubation, while that in medium A was 512.6 AU/ml. Maximum activity in medium B was approximately 5.1-fold higher than that in the basal medium. Although several reports on enhancement of pediocin production exist, significant increases (five fold) in pediocin production have not been reported (Mataragas et al., 2004; Todorov and Dicks, 2005; Kim et al., 2006).

In conclusion, the best conditions for the production of pediocin Iz3.13 were incubation at 30°C without agitation in a medium composed of 3.5% Proteose peptone No. 3, 3.5% Phytone peptone, 2.5% glucose, 1.5% $MgCl_2$, 3.0% beef extract and 0.25% K_2HPO_4.

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