

Research Article

Bacteriocin production and its crude characterization of lactic acid bacteria isolated from pickled *Garcinia schomburgkiana* Pierre

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Abstract

One hundred and sixty isolates of lactic acid bacteria (LAB) were isolated from natural lactic acid fermentation of pickled *Garcinia schomburgkiana* Pierre (Madan) and bacteriocin-producing LAB were then screened on de Man Rogosa and Sharpe agar (MRS) medium. Morphological, biochemical, physiological characteristics and carbohydrate fermentation were preliminary examined. Thirteen out of eighteen bacteriocin-producing isolates were identified as *Lactobacillus plantarum* 1, three isolates were *Lactobacillus brevis* 1, *Lactobacillus pentosus* and *Lactococcus lactis* ssp. *lactis* 1 and the remaining two isolates were unknown. Antimicrobial activity of the 16 identified bacteriocin-producing LAB isolates were quantified by agar well diffusion method. *Lactococcus lactis* ssp. *lactis* 1 provided the maximum crude bacteriocin production during fermentation which inhibited *Bacillus cereus* TISTR 687 and *Staphylococcus aureus* TISTR 118 but not against *Escherichia coli* TISTR 780. The inhibitory activities against *Bacillus cereus* TISTR 687 were more effective than that of *Staphylococcus aureus* TISTR 118. The optimal crude bacteriocin produced in MRS broth was achieved at the initial pH 6.5, 2% NaCl concentration (w/v) incubated at 35°C for 18 h. *Bacillus cereus* TISTR 687 inhibition was decreased by 80% of 50 mg/ml commercial nisin. False positive inhibition zone from non-proteolytic enzyme (catalase) indicated its proteinaceous nature by proteolytic enzyme inactivation such as alcalase, trypsin and papain. Heat stability of crude bacteriocin at 100°C and 90°C remained for 15 and 30 min, respectively, while no activity was observed at 121°C for 15 min. However, when crude bacteriocin was heated up to 60°C for 15 min, the antimicrobial activity was not significantly decreased when compared with unheated treatment ($p>0.05$). At pH 5.0 to 7.0 bacteriocin activity was stable but unstable at lower and higher pH 6.5 (control). Crude bacteriocin produced by *Lactococcus lactis* ssp. *lactis* 1 possesses heat stability at pasteurization temperature in pickling process and provides good potential as a starter culture.

Keywords: *Lactococcus*, fermentation, heat stability, food-borne pathogens, madan, Thailand.

Introduction

Lactic acid bacteria (LAB) play an important role in various food fermentations including meat, dairy, vegetable and fruit products. Antibacterial substances against food spoilage and food-borne pathogens such as organic acids, hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins can be produced from LAB [1]. Bacteriocin is the proteinaceous antibacterial compounds and these are most efficient against bacteria closely related to the producer strain and/or Gram-positive bacteria [2, 3]. Bacteriocin-producing strains are, therefore, practically used as food preservative cultures for ensuring the safety of fermented food products [4]. *Garcinia schomburgkiana* Pierre (Madan) is a tropical fruit belonging to the Guttiferae family found in all parts of Thailand and has high nutritional value and medical properties [5]. As the fresh fruit is generally too sour to consume, preservation methods such as pickling are usually considered for product improvement. The processing of pickled Madan is still accomplished at the household level and fermented spontaneously. Little scientific information of natural LAB isolated from pickled Madan has been reported. In order to improve the quality and food safety of pickled Madan, the potential bacteriocin-producing LAB starter culture should be examined. The aims of the current study were to isolate and identify bacteriocin-producing LAB from naturally fermented Madan based on morphological, biochemical, physiological characteristics and carbohydrate fermentation. The optimal production and preliminary characterization of crude bacteriocin were also performed.

Materials and Methods

Sources of test microorganisms

The test microorganisms; *Bacillus cereus* TISTR 687, *Staphylococcus aureus* TISTR 118 and *Escherichia coli* TISTR 780 were obtained from the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR). All strains were kept at 4°C and subcultured every two weeks on nutrient agar (NA, Hi-media, Mumbai, India) slant. The test microorganisms used for screening of bacteriocin-producing LAB were propagated twice in nutrient broth (NB, Hi-media, Mumbai, India) at 37°C overnight. The optical density of cell suspension was adjusted to equal the McFarland No.1 (about 10⁸ cells/ml) and diluted 100 times for antimicrobial study.

Sampling methods

Eight samples of natural pickled *Garcinia schomburgkiana* pierre (Madan) were collected from local markets in Thailand. The two samples were obtained from markets at Nakhon Nayok province and six samples from markets at Nakhon Ratchasima province. All samples were kept at 4°C until use.

Isolation and screening of lactic acid bacteria strains

LAB were isolated from pickled Madan by spread plate technique. Serial dilutions were made and 0.1 ml of the appropriate diluted saline solution were spread on De Man, Rogosa and Sharpe agar (MRS agar, Hi-media, Mumbai, India) supplemented with 1% calcium carbonate (CaCO₃), incubated at 30°C for 48-72 h under anaerobic condition. Colonies with different morphological characteristics were randomly selected and isolated to obtain pure cultures. The purified isolates were kept on MRS slant agar at 4°C for routine use and preserved in 10% skim milk powder at -20°C for stock culture. All of the isolated strains were examined using Gram staining, catalase production and oxidation/fermentation test. All isolates considered as LAB were selected for further study.

Identification of lactic acid bacteria strains

Morphological shape and arrangement of the cell were observed. Biochemical and physiological characteristics such as gas production from 1% glucose (w/v) containing Durham tube and motility of all strains according to Bergey's Manual of Systematic Bacteriology (1986) were done. In addition, the growth at temperature 10, 25, 30, 35 and 45 °C, pH 4.4 and 9.6 and 4, 6.5, 8, 10 and 18% NaCl concentration (w/v) in MRS broth (Sharpe, 1979) were tested as well. Carbohydrate fermentation tests were carried out by API 50 CHL kits and interpreted using APILAB PLUS V.3.2.2 software (Biomérieux, Marcy-l'Étoile, France).

Preliminary test for bacteriocin-producing strain

The agar spot test was performed as described in Mourad *et al.* [6], with some modification. The LAB isolates were screened for antimicrobial activity against test microorganisms (*Bacillus cereus* TISTR 687, *Staphylococcus aureus* TISTR 118 and *E. coli* TISTR 780). 5 µl of overnight-LAB isolates MRS broth culture were spotted onto the surface of bacteriocin screening medium (BSM) and anaerobically incubated at 30 °C for 24 h. After surface drying, these plates were overlaid with 7 ml nutrient soft agar (0.7% agar) containing overnight test microorganisms and continuously incubated at 30 °C for 18-24 h. The isolate of which the inhibition zone was more than 10 mm (in diameter) was interpreted as the potential antimicrobial-producing strain. These isolates continuously confirmed their antimicrobial production by well diffusion method.

Well diffusion method

The well diffusion method for antimicrobial production as described by Xiraphi *et al.* [7] was performed. The cell-free supernatant (CFS) was obtained by centrifugation of 18 h-isolates in MRS broth (pH 6.5) at 10,000 ×g (Sorvall Legend Mach 1.6R, Thermo Fisher Scientific, Germany) for 15 min at 4 °C and adjusting the pH of the CFS to 6.5 by sterilized 1 M NaOH or 1 M HCl and finally treated with catalase enzyme (C-3515, Sigma, St. Louis, USA) at room temperature for 30 min. The CFS was sterilized through a 0.2 µm pore-size cellulose acetate filter. The 50 µl of CFS was applied into the holes (6 mm in diameter) of 1.5% nutrient agar seeded with test microorganisms. The plates were incubated at 37 °C for 18-24 h after pre-diffusion at 4 °C for 24 h. The bacteriocin activity was represented as diameter (mm) of inhibition zone surrounding the wells. The inhibition zone width of more than 8 mm was scored as positive. The isolates with the largest inhibition zone and the most sensitive test microorganisms were chosen for further study.

Effect of growth conditions on bacteriocin production

pH

MRS broth was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 using sterile 3 M of NaOH or HCl. 1% of 18 h selected-LAB isolate was inoculated into each initial pH of MRS broth and shaking incubated (Orbital Incubator S-150, Stuart Scientific, UK) at 30 °C, 100 rpm for 0, 6, 12, 18, 24, 36 and 48 h. The optimal initial pH for bacteriocin production was selected for further study.

NaCl concentration

MRS broth containing 0, 1, 2, 3, 4 and 5% NaCl concentration (w/v) was inoculated with 1% of 18 h selected-LAB isolate and shaking incubated (Orbital Incubator S-150, Stuart Scientific, UK) at 30 °C, 100 rpm for 0, 6, 12, 18, 24, 36 and 48 h. The best NaCl concentration for bacteriocin production was selected for further study.

Incubation temperature

1% of 18 h selected-LAB isolate in MRS broth was shaking incubated at 30, 35, 37 and 40°C, 100 rpm. The samples of each temperature were taken out at 0, 6, 12, 18, 24, 36 and 48 h.

Growth was observed at the end of the interval incubation periods. Bacteriocin activity was determined by well diffusion method as described previously compared with 50 mg/ml nisin (Sigma, St. Louis, USA).

Preliminary characterization of bacteriocin

Enzyme activities

Crude bacteriocin sensitivity to proteolytic and non-proteolytic enzymes was carried out. The crude bacteriocin was treated with a final concentration of 2 mg/ml of proteolytic enzyme including alcalase, papain and trypsin (Sigma, St. Louis, USA) in 0.01 M phosphate buffer, pH of 7.0 and catalase (Sigma, St. Louis, USA) as non-proteolytic enzyme. The samples were incubated at 37°C for 2 h and enzyme activities were then eliminated by heating at 100°C for 3 min. The residual bacteriocin activity was assayed.

Heat stability

The effect of temperature on bacteriocin activity was tested at 60°C to 100°C (at increments of 10°C) in a thermostatically controlled water bath and 121°C for 15 min in an autoclave (Sanyo Vertical Labo Autoclave, NB Scientific, Edison, NJ). The samples were immediately cooled to room temperature in ice slurry water before being tested for residual bacteriocin activity.

pH sensitivity

Variation pH of crude bacteriocin was adjusted from 1.0-14.0 (at increments of one pH units) and pre-incubated at room temperature for 2 h and neutralized to pH 6.5 and then tested for residual bacteriocin activity.

The residual bacteriocin activity was measured as the diameter of inhibition zone (mm) against the test microorganisms using well diffusion method.

Statistical analysis

A two-way analysis of variance (ANOVA) test was used to evaluate the differences between residual activity of each treatment and mean comparison ($P < 0.05$) was performed by Duncan's multiple range test. Bar symbols were represented as standard deviation (SD) of inhibition zone (mean values in duplicate). All the experiments were performed in duplicate and the results were displayed as the mean value.

Results and Discussion

Isolation and identification of lactic acid bacteria

A total of 160 isolates from naturally pickled Madan using MRS agar containing 1% CaCO₃ were Gram-positive, catalase-negative and fermentation of 1% glucose (w/v) which is considered as belonging to LAB. The growth of LAB in lower glucose concentration showed no production of the high organic acids which can affect growth of the test microorganisms. Eighteen out of 160 isolates showed antimicrobial activity against *B. cereus* TISTR 687, *S. aureus* TISTR 118 but not *E. coli*

TISTR 780. This result is in agreement with the previous work of Rattanachaikunsopon and Phumkhachorn [8]. The crude bacteriocin was inefficient to against *E. coli* TISTR 780, it might be the result from outer member of Gram-negative bacteria obstructed the site of bacteriocin action [9, 10]. The antimicrobial activity of the 18 isolates was confirmed by using the well diffusion agar test. Eight out of the 18 isolates demonstrated antimicrobial activity against *B. cereus* TISTR 687 and/or *S. aureus* TISTR 118. The NN-MD1-7 (NN-MD was represented by Nakhon Ratchasima-Madan) isolate was shown to produce the largest inhibition zone against *B. cereus* TISTR 687 (data not shown). Therefore, this isolate was selected for further studies and using only *B. cereus* TISTR 687 as the test microorganisms. Based on morphological, biochemical, physiological characteristics and some carbohydrate fermentation by the API 50 CHL kits (Table 1.), 13 isolates of group I was identified as *Lactobacillus plantarum* 1, group II, III and IV as 3 *Lactobacillus brevis*, *Lactobacillus pentosus* and *Lactococcus lactis* ssp. *lactis* 1, respectively.

The NN-MD1-7 isolate was identified as *L. lactis* ssp. *lactis* 1. The isolate of group IV of which potential starter culture was coccobacilli (Figure 1). In this study, bacteriocin effectiveness against *S. aureus* TISTR 118 seems variation might be due to the increased resistance to bacteriocin that has been reported for *S. aureus* may be the result of alteration of bacterial membrane composition [11]. Fleming [12]; Ruiz-Barba and Jiménez-Díaz [13] showed that *L. plantarum* was often found at the end of fermentation. On the contrary, this experiment found that *L. lactis* ssp. *lactis* 1 was detected at the end of fermentation agrees with the result of Rattanachaikunsopon and Phumkhachorn [8], who reported that *Lactobacillus lactis* subsp. *lactis* isolated from fermented green onion was shown to produce the largest inhibition zone against *Leuconostoc mesenteroides* TISTR 473.

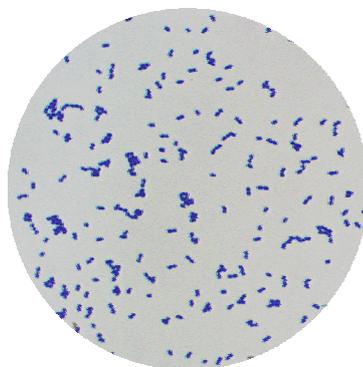


Figure 1. Gram stain of NN-MD1-7, bright field microscopy (×1000)

Table 1. Morphological, biochemical, physiological characteristics and carbohydrate fermentation of LAB isolated from pickled Madan.

Characteristics	Group I (13 isolates)	Group II (1 isolate)	Group III (1 isolate)	Group IV (1 isolate)
Gram staining	+	+	+	+
Cell shape	Rod	Rod	Rod	Cocccobacilli
Catalase test	-	-	-	-
Gas from glucose	-	-	-	-
Motility	-	-	-	-
Growth at or in				
10° C	+	-	+	+
25° C	+	+	+	+
30° C	+	+	+	+
35° C	+	+	+	+
45° C	+	+	+	-
pH 4.4	+	+	+	+ ^a
pH 9.6	+	+	+	+
4% NaCl	+	+	+	+
6.5% NaCl	+	+	+	+ ^a
8% NaCl	+	+	+	-
10% NaCl	+/- ^b	-	+	-
18% NaCl	-	-	-	-
Carbohydrates				
Glycerol	-	+	-	-
L-arabinose	+	+	+	-
D-xylose	+/- ^b	+	-	+
L-rhamnose	+/- ^b	+	-	-
Dulcitol	-	+	-	-
D-mannitol	+	+	+	+
D-lactose	+	+	+	-
D-melibiose	+/- ^b	+	+	-
Inulin	+/- ^b	+	+	-
D-melezitose	+	+	-	-
D-raffinose	+/- ^b	+	+	-
Gluconate	+	+	+	+
Identification (%)	<i>L. plantarum</i> 1 91.1-99.9	<i>L. pentosus</i> 99.9	<i>L. brevis</i> 1 99.8	<i>L. lactis</i> ssp. <i>lactis</i> 1 99.7

^a slight growth; ^b variation; + : positive; - : negative

Effect of growth conditions on bacteriocin production ***pH***

The initial pH of MRS broth medium affected crude bacteriocin production as presented in Figure 2. The inhibition zone directed variation with bacteriocin activity. The high bacteriocin activity of initial pH 6.5 was detected at 24 and 36 h. On the other hand, bacteriocin activity of initial pH 7.0 and 7.5 was decreased at the same time. When the bacteriocin activity reached the maximum value afterward it was decreased with prolonged incubation period. The initial pH 6.5 for 36 h resulted in the maximum bacteriocin activity, this initial pH was, therefore, selected for effect of NaCl concentration.

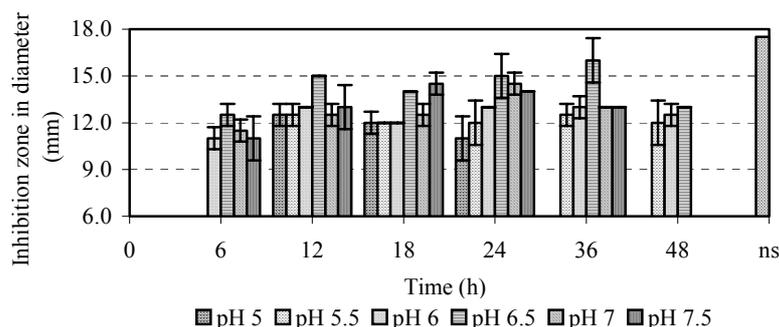


Figure 2. Effect of initial pH on bacteriocin production produced by NN-MD1-7.
(ns: 50 mg/ml of nisin)

Effect of NaCl concentration

Bacteriocin production of 0-3% NaCl concentration (w/v) was much more than that of 4-5% NaCl concentration (w/v) at the interval time for 48 h (Figure 3). The 2% NaCl concentration (w/v) for 12 h resulted in the maximum bacteriocin activity, thus the initial pH 6.5 and 2% NaCl concentration (w/v) were selected for incubation temperature condition.

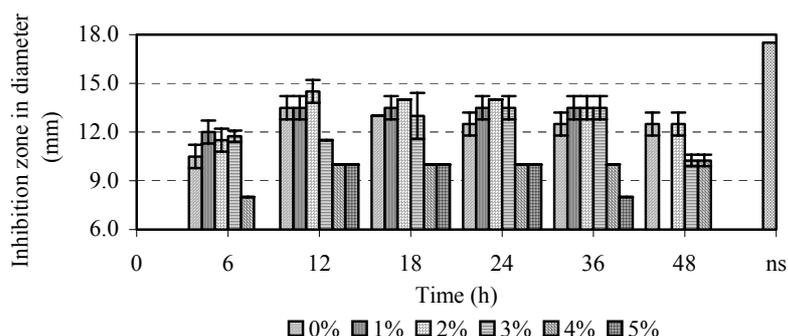


Figure 3. Effect of NaCl concentration on bacteriocin production produced by NN-MD1-7.
(ns: 50 mg/ml of nisin)

Effect of incubation temperature

Incubation temperature at 40°C achieved bacteriocin production only at 6 h. The maximum bacteriocin activity was found at 30°C, 35°C, 37°C and 40°C for 24, 18, 36 and 6 h, respectively (Figure 4).

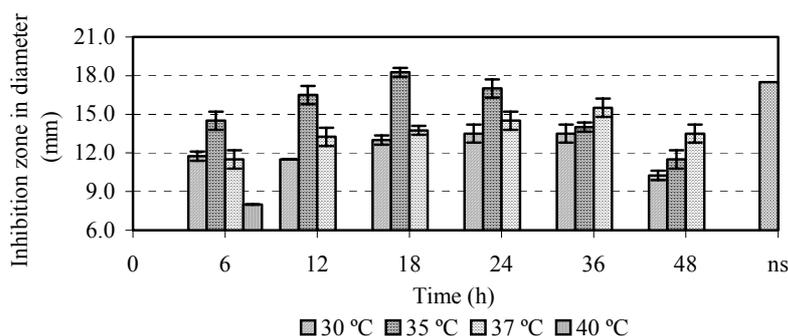


Figure 4. Effect of incubation temperature on bacteriocin production produced by NN-MD1-7.
(ns: 50 mg/ml of nisin)

Todorov and Dicks [14] have reported the highest bacteriocin activity produced by *L. pentosus* ST712B2 was found in MRS broth with initial pH of 6.5, 6.0 and 5.5. Whereas, the bacteriocin activity was decreased when this strain was grown in MRS broth with initial pH of 5.0 and 4.5. Todorov and Dicks [15] also showed that the maximum bacteriocin production of *L. plantarum* ST28MS was obtained from growth in MRS broth of the initial pH 6.0 and 6.5. The influences on bacteriocin production by LAB may be caused from various factors such as test microorganisms, variability and sensitivity of methods for determination of antimicrobial activity, purification condition of bacteriocin and experimental conditions [16]. Thus, the initial pH 6.5, 2% NaCl concentration (w/v) and 35°C incubation temperature for 18 h of optimal growth conditions incubation period were chosen for maximum bacteriocin production by *L. lactis* ssp. *lactis* 1 and its characterization. Results drawn from this experiment indicated that the crude bacteriocin produced in optimal conditions to *B. cereus* TISTR 687 inhibition effect was decreased 80% of 50 mg/ml commercial nisin (data not shown).

Preliminary characterization of bacteriocin

Crude bacteriocin totally lost antimicrobial activity when treated with proteolytic enzymes including alcalase, papain and trypsin. On the contrary, catalase enzyme unaffected to antimicrobial activity of crude bacteriocin, indicating that crude bacteriocin was the proteinaceous nature [17] and this agrees with the results of Ogunbanwo *et al.* [18] who reported that many non-protein antimicrobial agents could be produced from LAB, while only bacteriocin was proteinaceous in nature.

Heat treatment and pH effect of *L. lactis* ssp. *lactis* 1 on bacteriocin activity is presented in Table 2. The crude bacteriocin was thermostable since its activity was still found at 60°C to 100°C and 60°C to 90°C for 15 and 30 min, respectively, while any activity did not appear at 100°C for 30 min. The only residual activity of crude bacteriocin which heating up 60°C for 15 min was comparable to that of control (unheat treatment, $p > 0.05$). However, no significant residual activity was found at 60°C, 70°C and 80°C on heat treatments for 15 min ($p > 0.05$). Thus, the crude bacteriocin was stable on heat treatment up to 80°C for 15 min. The bacteriocin activity was completely lost at 121°C for 15 min. The thermostable feature in pasteurization level might be related to the molecular structure of the crude bacteriocin, usually composed by small peptides without tertiary structure [11].

Table 2. The effect of enzyme treatment, heat treatment and pH on residual bacteriocin activity produced by NN-MD1-7.

Treatments	Residual bacteriocin activity (%)	
Enzyme activities		
Alcalase	0	
Papain	0	
Trypsin	0	
Catalase	100 ^a	
Control	100 ^a	
Heat treatment		
	15 min	30 min
60 °C	95.58 ^{ab}	90.64 ^b
70 °C	94.40 ^b	88.99 ^b
80 °C	93.80 ^b	79.10 ^c
90 °C	83.59 ^c	56.03 ^d
100 °C	83.59 ^c	0
121 °C	0	ND
control	100 ^a	100 ^a
pH sensitivity		
3	52.19 ^d	
4	56.56 ^d	
5	93.48 ^b	
6	100 ^a	
7	95.69 ^{ab}	
8	67.38 ^c	
9	54.34 ^d	
control	100 ^a	

ND = Not determined; Values with different superscripts are statistically significant ($P < 0.05$) (compared within column of each treatments)

The residual activity remained at pH 3.0 to 9.0 range meanwhile declined activity when treated with those exterior ranges. The high residual activity was found in the ranges of 93-100% at pH 5.0 to 7.0 and maximum activity was shown at pH 6.0. When the crude bacteriocin was adjusted to pH range 1.0 to 4.0 and 10.0 to 14.0, it was difficult to neutralize to pH 6.5 for bacteriocin assay because of dilution of crude bacteriocin. Therefore, no activity on this pH ranges was detected as shown in Table 2. The crude bacteriocin was detected in acidic condition (pH 3.0 to 6.0) but not in the alkaline condition, due to alkali lysis [3]. This finding suggested that the crude bacteriocin or bacteriocin-producing bacteria should be used in combination with other preservation methods such as pasteurized thermal processing to improve food safety and quality [19].

Conclusion

This study demonstrated that *L. lactis* ssp. *lactis* 1 isolated from the naturally pickled Madan could inhibit the growth of food borne pathogens including *B. cereus* TISTR 687 and *S. aureus* TISTR 118. The initial pH 6.5, 2% NaCl concentration (w/v) and 35 °C incubation temperature was found to be the most effective for bacteriocin production by *L. lactis* ssp. *lactis* 1. The crude bacteriocin was proteinaceous in nature, stable in pH range 5.0 to 7.0 and resistant to heat at 60 °C for 15 min compared with control. Therefore, *L. lactis* ssp. *lactis* 1 should be considered for its potential as a starter culture and its crude bacteriocin was heat stable compared to pasteurization temperature in the pickling process.

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