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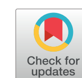
Short communication

Development of a new protocol for freeze-drying preservation of *Pseudoalteromonas nigrifaciens* and its protective effect on other marine bacteria



Zheng Zhang [a,b,1], Yong-xiang Yu [a,1], Yin-geng Wang [a,b,*], Xin-xian Wei [c], Mei-jie Liao [a,b], Xiao-jun Rong [a,b], Jing Chen [a]

[a] *Key Laboratory of Maricultural Organism Disease Control, Ministry of Agriculture and Rural Affairs, Yellow Sea Fisheries Research Institute, Chinese Academic of Fishery Sciences, Qingdao 266071, PR China*

[b] *Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, PR China*

[c] *Guangxi Academy of Fishery Sciences, Guangxi Key Laboratory of Aquatic Genetic Breeding and Healthy Aquaculture, Nanning 530021, PR China*

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ABSTRACT

Background: Freeze-drying is known as one of the best methods to preserve bacterial strains. Protectant is the key factor affecting the survival rate of freeze-dried strains. In addition, salinity, bacterial suspension concentration, drying time, and other factors can also affect the survival rate of strains to varying degrees. At present, there are relatively few studies on freeze-drying preservation of marine bacteria. In the present study, we performed the freeze-drying protectant screening and optimized the preservation conditions for *Pseudoalteromonas nigrifaciens*, which is widely distributed in marine environment. The protective effects of the screened protectants were verified by 18 other marine bacterial strains.
Results: The results indicated that the combination of 5.0% (w/v) lactose, 5.0% (w/v) mannitol, 5.0% (w/v) trehalose, 10.0% (w/v) skim milk powder, 0.5% (w/v) ascorbic acid and 0.5% (w/v) gelatin was the best choice for the preservation of *P. nigrifaciens*. The suggested salinity and concentration of initial cell suspension were 10 g/L NaCl and 1.0×10^9 CFU/mL, respectively. Furthermore, stationary-phase cells were the best choice for the freeze-drying process. The highest survival rate of *P. nigrifaciens* reached 52.8% when using 5–10% (w/v) skim milk as rehydration medium. Moreover, the other 18 marine strains belonging to *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Planomicrobium*, *Edwardsiella*, *Enterococcus*, *Bacillus*, and *Saccharomyces* were freeze-dried under the abovementioned conditions. Their survival rates were 2.3–95.1%.
Conclusion: Collectively, our results supported that the protectant mixture and parameters were beneficial for lyophilization of marine bacteria.
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1. Introduction

Long-term preservation of bacterial strains plays a fundamental role in scientific research and application of microbiology. In general, ultra-low temperature is commonly used for the preservation of microorganisms [1]. However, it has many intrinsic drawbacks such as short storage time and necessity of regular subculture. Continuous subculture in vitro would cause changes in physical and chemical characteristics [2]. Freeze-drying is generally accepted as an effective technique for long-term preservation of biological materials such as eukaryotic cells [3], bacteria [4], viruses [5], and functional proteins [6]. Biological cells have to experience two types of environment stress, freezing and drying, leading to inevitable damages. These damages include mechanical damage, solution effect damage, membrane permeability change, proteins denaturation, pH dynamic balance disturbance, cell membrane fatty acid composition and permeability change.

To obtain the highest cell survival rate, many factors during freeze-drying process have been widely investigated, such as damage mechanisms [7], protectants or additives [8,9], freeze-drying condition, and parameter optimization [10]. The protectant composition is usually considered as the most important factor to affect the survival rate of

* Corresponding author.
E-mail address: wangyg@ysfri.ac.cn (Y. Wang).
[1] These authors contributed equally to this work
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cells during freeze-drying. Appropriate protectants can reduce cell damage during freeze-drying and rehydration, maintain their physiological activity and store stability during long-term preservation.

There are various microbial resources in marine environment. Marine microbial strains collection, identification and preservation can provide more abundant material sources for humans. The establishment of freeze-drying preservation technology for marine bacteria stain is the prerequisite for making full use of marine microbial resources. To date, most studies on microbial freeze-drying have focused on terrestrial bacteria, such as *Streptococcus* [11], yeast [12] and *Lactobacillus* [13]. However, some important marine pathogenic bacteria, such as *Vibrio*, exhibit low survival rate after lyophilization [14]. Because of the disparities between marine and terrestrial environments, especially osmolality and salinity, the factors that impact the freeze-drying preservation of marine bacteria may be different.

P. nigrifaciens belonging to *Pseudoalteromonas* is widely distributed in ocean [15,16]. It has multiple environmental adaptive capacities, and can secrete various extracellular active substances [17] to ensure its survival and play important roles in marine ecology system. Some strains are conditional pathogens that can cause marine diseases of plants and animals, such as sea tangle [18], sea cucumber [19], fish [20] and others. However, some of them are used as potential probiotics in aquaculture [21]. In the present study, we aimed to optimize protectant composition, bacterial suspension and parameters of *P. nigrifaciens* in freeze-drying process. Our findings provided a new choice for lyophilization of marine bacteria.

2. Materials and methods

2.1. Bacterial strain

The strain of *P. nigrifaciens* used in this study was obtained from Yellow Sea Fisheries Research Institute, China, which has been reported as farmed *Apostichopus japonicas* pathogen [22]. After cultivation in trypticase soy broth (TSB), the cells were harvested by centrifugation and washed with 15 g/L NaCl solution twice. Subsequently, the cell pellet was resuspended in 15 g/L NaCl solution to obtain bacterial suspension of different concentrations.

2.2. Protectant screening and orthogonal test

Eight types of single protectants, including trehalose, mannitol, lactose, fructose, glucose, skim milk, ascorbic acid and gelatin, were screened in the first step. They have been reported as common protectants for freeze-drying [13,23,24,25,26]. In this study, all the protectants were dissolved in 15 g/L NaCl solution to set five gradient concentrations ranging from 5% to 20% (Fig. 1). Ascorbic acid was sterilized through a 0.22-μm filter membrane. Skim milk was sterilized by boiling. Moreover, the other protectants were sterilized at 106°C for 30 min. Adjusted orthogonal combination test was performed for selection of single protectants. Its array of L_{16} ($4^4 \times 2^2$) was set to analyze protectant composition (Table S1). Trehalose (0.0%, 5.0%, 7.5% and 10.0%, w/v), mannitol (0.0%, 5.0%, 7.5% and 10.0%, w/v), lactose (0.0%, 5.0%, 7.5% and 10.0%, w/v) and skim milk powder (0.0%, 5.0%, 10.0% and 15.0%, w/v) of different concentrations were prepared. Protectant mixtures were sterilized at 106°C for 30 min first, and then ascorbic acid (0.0% and 0.5%, w/v) and gelatin (0.0% and 0.5%, w/v) were added as antioxidant and excipient, respectively.

2.3. Lyophilization and the protection effect

The selected protectants were dissolved in 5 g/L NaCl solution and mixed with equal volume of *P. nigrifaciens* suspension (1.0 $\times$ 10^9 CFU/mL) in serum bottle. The eutectic point of the mixture was determined by lyophilizer (Martin Christ LyoLog-32). Based on the measured eutectic point, the freeze-drying process included the following steps. Mixture (1 mL) was frozen at -80°C for 6 h, followed by freeze-drying at -36°C, 0.200 mbar for 18 h and -42°C, 00.100 mbar for 2 h. Ice nucleation, in this situation, could be significantly reduced during freeze-drying [11]. The serum bottles were capped in freeze dryer chamber. Freeze-dried bacterial powder was activated after 24 h. The powder was rehydrated with 1 mL sterile distilled water, and the survival rate was determined by 10-fold gradient dilutions.

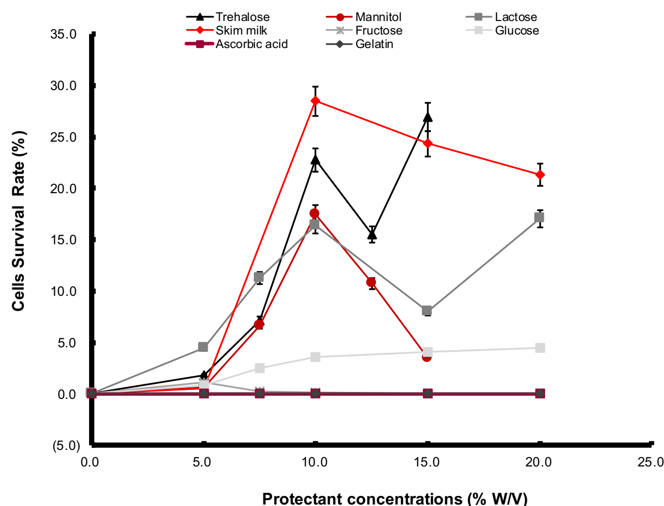

Fig. 1. The survival rates of *P. nigrifaciens* in the presence of different single protectants. Standard deviations were added in the line chart.

2.4. Salinity, initial cell concentration, and bacterial strain growth stage optimization

Salinity of mixture, initial cell concentration and bacterial growth stage of *P. nigrifaciens* suspension before freeze-drying were also considered for optimization. The NaCl concentrations were set to 0, 5, 10, 15, 20 and 25 g/L. Different cell concentration gradients, including 5.0×10^9, 1.0×10^9, 5.0×10^8, 5.0×10^7 and 5.0×10^6 CFU/mL, were prepared by diluting with 15 g/L NaCl solution. Five time points were selected according the growth curve of *P. nigrifaciens* (Fig. S1), including the 9th h (early stage of logarithmic growth phase), 12th h (logarithmic growth phase), 16th h (the critical point of logarithmic growth phase and stationary phase), 20th h (stationary phase) and 26th h (stationary phase). Rehydration was performed at 24 h after freeze-drying was completed. Eight rehydration media, including distilled water, sterile sea water, 0.5%, 1.0%, 1.5% (w/v) NaCl solution, and 1.0%, 5.0%, 10.0% (w/v) skim milk, were prepared. Next, 1 mL rehydration medium was added to the bacterial powder, and the cell survival rate was then determined.

2.5. The freeze-drying of other marine bacteria

The other 18 marine bacterial strains belonging to *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Planomicrobium*, *Edwardsiella*, *Enterococcus*, *Bacillus*, and *Saccharomyces* were selected (Table S2) to evaluate the protective effect of protectants in freeze-drying process. These strains were isolated from mariculture animals or environment and preserved in Yellow Sea Fisheries Research Institute, China. Cell suspensions of the abovementioned strains (1.0 $\times$ 10^9 CFU/mL) were obtained by using 15 g/L NaCl solution. The suspensions were mixed with equal volume of protectants and frozen at -80°C for 6 h, followed by freeze-drying process as described above. Finally, the powder was rehydrated with 10% (w/v) skim milk, and the cell survival rate was determined.

2.6. Statistical analysis

All statistical analyses were performed using SPSS v.19.0 software. Data were presented as mean ± standard deviation (SD). A two-tailed P value of less than 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Single protectant screening

Fig. 1 shows the protective effects of eight single protectants at different concentrations. No living bacteria were detected in the presence of ascorbic acid and gelatin. Lower than 5% *P. nigrifaciens* cells survived in the presence of glucose and fructose at different concentrations. However, the cells survival rate was 28.53% in 10% (w/v) skin milk and 26.95% in 20% (w/v) trehalose. Regarding the other two protectants, the maximum survival rates were 17.05% in 15% (w/v) lactose and 17.53% in 10% (w/v) mannitol. The results indicated that skim milk and trehalose as single protectants had the best protective effects on *P. nigrifaciens*.

Saccharides can help the cell to avoid enzyme leakage after the cell wall is broken [23]. Because of different physical and chemical characteristics, such as bonding ability and reducing activity, the protective effect of monosaccharide (reducing sugar) is usually lower than that of disaccharide. Previous studies have indicated that the protective effect of nonreducing disaccharides is related to their interactions with protein and membranes [24,25]. Skim milk exhibited a better protective effect on *P. nigrifaciens* upon freeze-drying than other single protectants. This finding might be attributed to its two major components, protein and saccharides. Proteins can react with bacterial structural protein and form a biofilm on the surface of bacteria. Ascorbic acid alone or in combination with excipients can protect *Lactobacillus* from freeze-drying and long-term preservation [13]. Because the limited protective effect, gelatin is usually used as a better excipient [26].

3.2. Optimized protectant composition and its eutectic point

A total of 16 protectant mixture groups at various protectant concentrations were investigated in the present study. Table S1 shows that the survival rates of *P. nigrifaciens* were lower than 0.01% in groups 1, 12 and 14. Meanwhile, the highest survival rate of 29.93% was found in group 10. All groups showed a lower survival rate compared with 10% (*w/v*) skim milk alone except for groups 6 and 10. Variance analysis using SPSS v.19.0 indicated that skim milk, ascorbic acid and mannitol had significant effects on the survival rate of *P. nigrifaciens* ($p < 0.05$). Based on F value shown in Table 1, skim milk had the highest impact, followed by ascorbic acid, mannitol, lactose, gelatin and trehalose. Table 2 lists the survival rates in the presence of different protectants at various concentrations. According to the orthogonal analysis, the optimal protectant mixture consisted of 5.0% (*w/v*) lactose, 5.0% (w/v) mannitol, 5.0% (w/v) trehalose and 10.0% (w/v) skim milk. Furthermore, 0.5% (w/v) ascorbic acid and 0.5% (w/v) gelatin were suggested to be added as antioxidant and excipient, respectively. The eutectic point of this mixture was -25.8°C (Fig. S1).

Table 1
Orthogonal variance analysis for different protectants based on the cell survival rate.

	Type III sum of squares	df	MS	F	P
Corrected Model	2075.183	14	148.227	644.819	0.031
Intercept	5461.817	1	5461.817	23,760.021	0.004
Mannitol	162.254	3	54.085	235.279	0.048
Lactose	117.151	3	39.050	169.878	0.056
Trehalose	21.289	3	7.096	30.871	0.131
Skim milk	1572.645	3	524.215	2280.442	0.015
Ascorbic acid	174.747	1	174.747	760.184	0.023
Gelatin	27.097	1	27.097	117.878	0.058

Note: According to the P value and F value, skim milk shows significant difference and highest impact of protective effect during freeze-drying.

Table 2
The mean and significant analysis of different protectants based on cell survival rate.

Level	Mean and significant analysis					
	Lactose	Mannitol	Trehalose	Skim milk	Ascorbic acid	Gelatin
1	13.990[Ab]	19.024[Aab]	17.551[Aa]	2.979[Bc]	21.781[Aa]	17.175[Aa]
2	19.332[Aa]	23.099[Aa]	20.051[Aa]	16.678[ABb]	15.171[Ab]	19.777[Aa]
3	21.250[Aa]	17.551[Abc]	19.092[Aa]	27.466[Aa]	–	–
4	19.332[Aa]	14.229[Abc]	17.209[Aa]	26.781[Aa]	–	–
5	0.494[Aa]	0.783[Aa]	0.090[Aa]	0.738[Aa]	–	–

Note: [a,b] Row mean significant difference; for lactose, mannitol and trehalose, level 1 to 4 were corresponding to the concentrations of 0.0%, 5.0%, 7.5% and 10.0% (w/v), respectively; for skim milk, they were 0.0%, 5.0%, 10.0% and 15.0% (*w/v*); for ascorbic acid and gelatin, level 1 and 2 were 0.0% and 0.5% (*w/v*), respectively; –, no data.

Bacteria suffer from serious damage during the freeze-drying process. Many studies have confirmed that the combinations of protectants can improve cell survival rate after freeze-drying compared with single protectants [11,27,28]. However, this finding may be species-dependent. For example, in *Pseudomonas chlororaphis*, the cell survival rate using protectant mixtures is lower than that obtained using disaccharides (lactose, sucrose and trehalose) alone after freeze-drying process [29].

3.3. The effects of salinity, initial cell concentration, growth stage, and rehydration medium

Fig. 2a shows that when 15 g/L NaCl solution was used for the preparation of bacterial suspension, the maximum survival rate of *P. nigrifaciens* was 40.3%. For the initial bacterial concentration, the cell survival rate was increased up to maximum of 36.9% when the initial cell concentration of bacterial suspension was 1.0×10^9 CFU/mL (Fig. 2b). Furthermore, with the increase in salinity or initial cell concentration, the survival rate of *P. nigrifaciens* was gradually decreased. Previous studies have reported that bacteria can sense environmental changes, respond appropriately, and enhance their tolerance to adverse environment [30,31]. Salinity is the key factor for the maintenance of cellular osmotic pressure. It is essential to optimize the protectant salinity to obtain high survival rate of marine bacteria after freeze-drying process. The results confirmed that the survival rate of *P. nigrifaciens* was decreased with the increase of salinity, which was probably attributed to structural damage of cells or protein denature caused by salt crystals.

Proper cell concentration can keep the best protectant distribution in intercellular space and achieve the maximum protective effects during freeze-drying process. The proportion of protectant and bacterial suspension has strong effect on the protectant distribution around bacterial cell, and affects the survival rate after freeze-drying [32]. Furthermore, appropriate initial cell concentration for freeze-drying may vary due to different bacterial species. A previous study indicated that the optimal initial cell concentration is between 1×10^9 to 1×10^{10} CFU/mL for *Pseudomonas chlororaphis* [29], while it is 1–3 $\times 10^{10}$ CFU/mL for *Vibrio anguillarum* [33]. We found that the cell survival rate was increased up to maximum when the initial cell concentration of *P. nigrifaciens* suspension was 1.0×10^9 CFU/mL, and the survival rate of *P. nigrifaciens* was gradually decreased with the increase of initial cell concentration.

Fig. S2 shows the growth curve of *P. nigrifaciens*. The highest cell survival rate was 41.5% when the 22th-h cells were used to prepare bacterial suspension. The lowest survival rate was found from cells in the logarithmic phase (less than 30%) (Fig. 2c). As a result, stationary phase cells were suggested to be optimal choice for *P. nigrifaciens* during freeze-drying. The best rehydration effects were achieved when 5–10% (*w/v*) skim milk was used as rehydration medium. Under this condition, the survival rate of *P. nigrifaciens* was higher than 38.0%. Sterilized sea water showed the worst rehydration effect, with

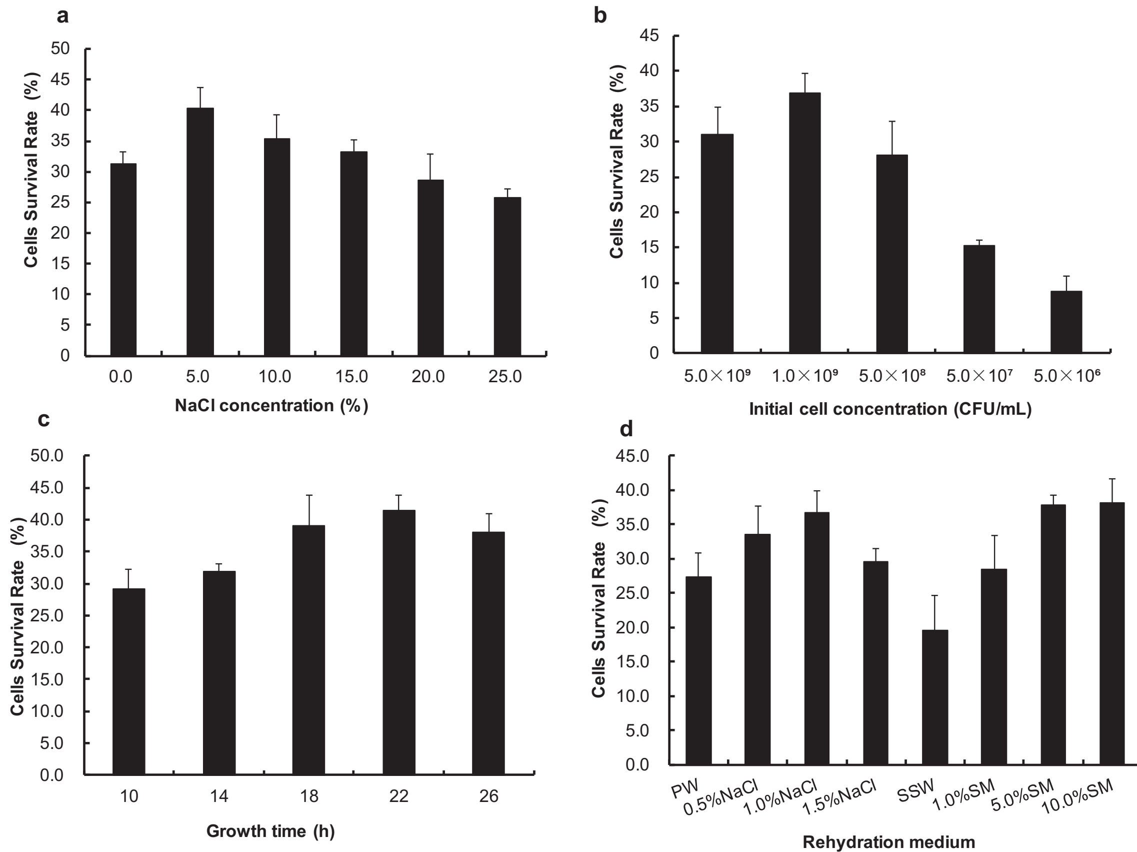

Fig. 2. The survival rates of *P. nigrifaciens* with different freeze-drying parameters. Error bars represent standard deviation (SD) from the mean of triplicate tests. (a): different NaCl concentrations in protectant mixture; (b): different cell concentrations of initial bacterial suspension; (c): different growth times of bacteria; (d): different rehydration media.

a cell survival rate of only 19.6% (Fig. 2d). Therefore, 5–10% (w/v) skim milk was recommended as the best choice of rehydration medium.

The bacterial shape, adhesion ability, biochemical reaction or other characteristics are affected by growth phase [34,35]. The prokaryotes in the stationary phase can retain the highest survival rate after freeze-drying [36]. In this study, *P. nigrifaciens* in the stationary phase also exhibited the best protective effect, while the cells in the logarithmic phase showed the lowest survival rate. This finding was potentially attributed to the fact that the cells in the stationary phase had stabilized morphological and physiological characteristics, while cells in the logarithmic phase were growing fast and sensitive to adverse environment. The last but not the least, appropriate rehydration condition could repair some nonfatal injuries of cells during freeze-drying. The same bacterial strains have different survival rates after freeze-drying due to the difference in rehydration medium [37].

3.4. The protective effects of other marine bacteria

The survival rates of the other 18 marine bacteria ranged from 2.3% to 95.1% after freeze-drying using the abovementioned protectant mixture and parameters (Table S2). *Bacillus*, *Saccharomyces*, and *Candida* achieved the maximum survival rates of about 90.0%. *Vibrio* showed the lowest protective effects with an average survival rate of less than 10%. The cell survival rates of *Pseudoalteromonas*, *Photobacterium*, *Planomicrobium*, *Edwardsiella*, and *Enterococcus* ranged from 34.3% to 68.4%. These results supported that the protectant mixture and parameters optimized in this study would be good choices for freeze-drying preservation of marine bacteria.

4. Conclusions

Our results support that marine bacteria can reach high cell survival rate after freeze-drying treatment. In order to obtain the best protection effect of lyophilization, appropriate protectant formula screening is necessary. Some parameters such as salinity, initial cell concentration, and strain growth stage must also be considered for optimization. In this study, we suggested that the mixture of 5.0% (*w*/*v*) lactose, 5.0% (w/v) mannitol, 5.0% (w/v) trehalose, 10.0% (w/v) skim milk, 0.5% (w/v) ascorbic acid and 0.5% (w/v) gelatin was the optimal protectant composition for *P. nigrifaciens* upon freeze-drying. The salinity and cell concentration in protectant-bacterial suspension mixture should be set as 10 g/L NaCl and 1.0×10^9 CFU/mL, respectively. Furthermore, the cells in the stationary phase and 5–10% (w/v) skim milk were recommended to prepare bacterial suspension and to rehydrate freeze-dried powder, respectively.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary material

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