



# Production of Bacteriocin-like Substances by *Bacillus* Spp. JY-1 in Soy Whey

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**Abstract:** Soy whey is a by-product from the processing of soybean products, which is generally discarded and considered as waste. In recent years, a large number of bacteriocins produced by bacteria have been described. However, the production of bacteriocins in soy whey has not yet reported. Bacteriocin-like substance producing *B. spp* JY-1 was isolated from Chinese traditional fermented soybean (*douchi*) in previous study. In present study, the antimicrobial spectrum, and the effect of enzymes, pH and heat on the antibacterial activity of bacteriocin-like substance produced by *B. spp* JY-1 were evaluated. Then, the effects of supplement of carbon and nitrogen sources on the production of bacteriocin-like substances in soy whey were also investigated. Results obtained indicated that bacteriocin-like substance in cell-free supernatant of JY-1 exhibited broad inhibitory spectrum both against some food-borne pathogens. The bacteriocin-like substance JY-1 was sensitive to trypsin and pepsin, but stable between pH 2.0-10, and heat resistance (65-105°C). In addition, the production of bacteriocin-like substance JY-1 started at the early exponential phase and reached its maximum at the stationary phase. The antimicrobial activity of cell-free supernatant of JY-1 cultured in soy whey was observed. The supplement of soluble starch or beef extract in soy whey yielded a higher production of bacteriocin-like substance. The results indicated that the bacteriocin-like substance JY-1 may be a potential candidate for alternative agent to control important food pathogens, and the soy whey has potential for production of bacteriocins.

**Keywords:** *B. Spp* JY-1, Bacteriocin-like Substance, Antimicrobial Activity, Soy Whey

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## 1. Introduction

Bacteriocins are ribosomally synthesized antibacterial peptides that possess antagonistic activity toward closely related strains, while its producer cells are immune to their own bacteriocins [1]. Numerous bacteriocins from Gram-positive bacteria, mainly lactic acid bacteria (LAB) and *Bacillus* spp.

*Bacillus* species are also considered to be good producers of antimicrobial compounds consisting of more than different types such as bacteriocins, bacteriocin-like substances, and antibacterial lipopeptides [2], and bacteriocins from *Bacillus* exhibit distinct diversity in terms of their inhibitory activities and pH resistance [3]. In particular, *Bacillus* strains are

generally known to be thermo-tolerant and spore-forming species that show rapid growth in liquid culture and are widely distributed in environment, such as soil, fermented foods. There is growing interest in recent research on the isolation and identification of the bacteriocin-producing *Bacillus* spp. from the fermented foods. In the study conducted by Lim [4] revealed that *Bacillus amyloliquefaciens* J4 isolated from Korean traditional fermented soybean paste had a broad spectrum of antibacterial activity against various food-borne pathogens, especially the *Listeria monocytogenes*, however, the antimicrobial activity of bacteriocin J4 was completely abolished by treatment with proteolytic enzymes. Previous study reported that *B. amyloliquefaciens* EMD17 isolated from *Cheonggukjang* strongly inhibited growth of *L. monocytogenes*, *Bacillus cereus*, and the antimicrobial

substance was non-proteinous nature, and several genes encoding lipopeptides were detected [5]. *Bacillus licheniformis* is a gram-positive, spore-forming soli bacterium that is used for the production of enzymes, antibiotics, biochemical and consumer products on an industrial scale [6, 7]. *B. licheniformis* strains isolated from papaya [8] and sediments [9] exhibited a wide spectrum of antimicrobial activity against several bacteria.

Soybean is an important type of legume that has been used for a long period as a protein source in Asia countries [10]. The consumption of soy foods is increasing around the world due to its rich nutritional value and several potential health benefits to humans. Soy whey is a by-product from the preparation of soybean products such as tofu, and soy protein isolates, among others. Large amounts of soy whey are generally discarded and considered as waste, which have aggravated burden of the industry on sewage treatment and also a waste of this resource [11]. The nutrient content in soy whey, which consists of carbohydrates, nitrogen compounds and minerals, supports microbial growth [12]. Soy whey has been reported to be able to support the growth of bacteria. Soy whey supplemented with various nitrogen sources was used to grow lactic acid bacteria for production of nisin [13] and tofu coagulant [14], enhancing the angiotensin-I converting enzyme (ACE)-inhibitory activity [15], antioxidant capacity [16]. Until now, the growth of *Bacillus* in soy whey had never been reported.

In another recent study, *B. spp* JY-1 with strong antimicrobial activity was isolated from traditional fermented soybean (douchi) in China. The aim of this study was to evaluate the biochemical properties of the antimicrobial compounds of JY-1. In addition, the production of the bacteriocin-like substances in soy whey fermented with *B. spp* JY-1 was investigated, and the effects of nutrients on the production of bacteriocin-like substances are discussed.

## 2. Materials and Methods

### 2.1. Bacterial Strains and Growth Conditions

Strain JY-1 reported for the first time in the literature to produce bacteriocin-like substances was isolated from *douchi*, a Chinese traditional fermented soybean and classified as *Bacillus* spp. based on phenotypic. Strain JY-1 were grown in BHI medium at 37°C in a rotary shaker at 150 rpm. The other strains used as indicator organisms were grown using the appropriate media and temperature as indicated in Table 1. Bacteria were kept as frozen cultures in 20% (v/v) glycerol at -80°C.

### 2.2. Determination of Antimicrobial Spectrum

Bacteriocin-like substances from strain JY-1 were tested for the antimicrobial spectrum against a wide range of bacteria comprising both Gram-positive and Gram-negative members, propagated in appropriate media as listed in Table 1. Antibacterial activity was determined by the well-diffusion method using *L. monocytogenes* 54002 as the indicator strain

[6]. The inhibition zone diameters were measured with a Vernier caliper.

### 2.3. Stability of the Antimicrobial Substance Against Enzymes, pH and Heat Treatment

Effect of enzymes, pH and heat on the antibacterial activity of *Bacillus* spp. JY-1

was examined as described by Liu *et al.* [8]. The concentrated supernatant of *Bacillus* spp. JY-1 was treated with trypsin (Amresco, USA), pepsin (Amresco, USA), neutral proteinase (Amresco, USA) and proteinase K (Sigma, USA). To evaluate the effect of heat on bacteriocin activity, the concentrated supernatant of *Bacillus* spp. JY-1 was heated at temperature of 65, 85, 105 and 121°C for 30 min, respectively. The sensitivity of the active substances to different pH values was estimated by adjusting the pH of the *Bacillus* spp. JY-1 cell-free supernatant to between 2 and 10 using 5 M NaOH or 5 M HCl. The experiments were carried out in duplicate using the untreated supernatant as the control.

### 2.4. Bacteriocin-like Substances Production During Growth

BHI broth was inoculated with 1% (v/v) of an overnight culture of JY-1 and incubated at 37°C in a rotary shaker at 150 rpm. Changes in optical density (600 nm) were recorded every three hour for 24 h. Antimicrobial activity in the cell-free supernatant was measured at the same time intervals.

### 2.5. Production of Bacteriocins by *Bacillus* Spp. JY-1 in Soy Whey

Fresh soy whey were filtered and then autoclaved (121°C, 20 min). To test the effect of carbon and nitrogen sources on the production of bacteriocin-like substances by JY-1 in soy whey, each carbon source (glucose, lactose, fructose, galactose, sucrose, maltose, soluble starch) or nitrogen source (tryptone, meat extract, yeast extract) was added to MRS at 2% level, respectively. The soy whey based mediums were inoculated with 2% (v/v) of an 24-h-old culture of strain JY-1. Antimicrobial activity in the cell-free supernatant of each medium were measured.

## 3. Results

### 3.1. Antimicrobial Spectrum of Bacteriocin JY-1

In order to study the antimicrobial spectrum of bacteriocin-like substances of JY-1, several LAB strains and food-borne pathogenic bacteria comprising both Gram-positive and Gram-negative members were tested (Table 1). Bacteriocin-like substances JY-1 showed a moderate spectrum of activity against most of tested strains including the genera *Listeria*, *Salmonella*, *Shigella*, *Staphylococcus*, *Clostridium* and *Bacillus*, especially *L. monocytogenes*, which was foodborne pathogenic bacteria. Besides pathogenic bacteria, *Bifidobacterium animalis* and *Lactobacillus fermentum* were also sensitive to bacteriocin-like substances JY-1. The results showed that the

antimicrobial compounds in JY-1 supernatant exhibit a broad spectrum of antimicrobial activity against most indicator strains.

**Table 1.** Spectrum of antimicrobial activity of bacteriocin JY-1.

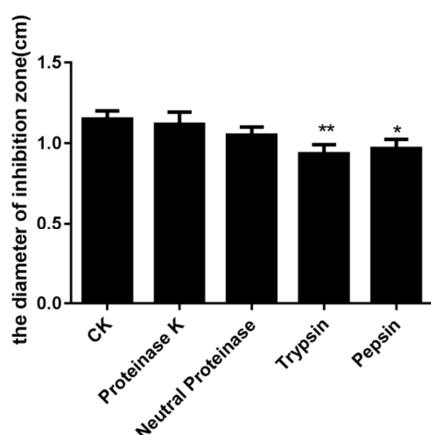
Indicator organism	Source <sup>a</sup>	Medium-incubation temperature (°C)	Sensitivity <sup>b</sup>
<i>Staphylococcus aureus</i>	ATCC 25923	LB, 37°C	++
<i>Shigella flexneri</i>	ATCC 12022	LB, 37°C	++
<i>Shig. bagdii</i>	CMCC 51218	LB, 37°C	+
<i>Shig. sonnei</i>	CMCC 54005	LB, 37°C	+
<i>Bacillus subtilis</i>	CMCC 63501	LB, 37°C	++
<i>Candida albicans</i>	CMCC (F) 98001	LB, 37°C	++
<i>Clostridium perfringens</i>	CICC 22949	LB, 37°C	++
<i>Salmonella typhimurium</i>	ATCC 14028	LB, 37°C	+
<i>Salmonella typhimurium</i>	CGMCC 1.1174	LB, 37°C	++
<i>Salmonella sp.</i>	CGMCC 1.1552	LB, 37°C	++
<i>Salmonella anatum</i>	CICC 21498	LB, 37°C	++
<i>Salmonella choleraesuis subsp.</i>	CGMCC 1.1859	LB, 37°C	++
<i>Listeria monocytogenes</i>	CMCC 54002	LB, 37°C	+++
<i>Listeria monocytogenes</i>	ATCC 15313	LB, 37°C	++
<i>Micrococcus luteus</i>	CMCC 28001	LB, 37°C	+
<i>Lactobacillus fermentum</i>	JCM 1173	MRS, 37°C	++
<i>Bifidobacterium animalis</i>	CGMCC 1.2268	MRS, 37°C	+++
<i>Lactobacillus plantarum</i>	CICC 6240	MRS, 37°C	+

<sup>a</sup>ATCC, American Type Culture Collection; CGMCC, China Center of General Microbial Culture Collection; CICC, China Center of Industrial Culture Collection; CMCC, National Center For Medical Culture Collections; JCM, Japan Collection of Microorganisms; NBRC, NITE Biological Resource Center.

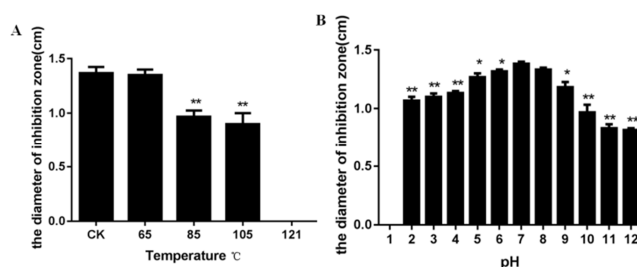
<sup>b</sup>Inhibition zone (mm): +++, 15-25 mm; ++, 8-14 mm; +, 1-7 mm; -, no inhibition.

### 3.2. Effects of Enzymes, pH and Heat on Bacteriocin JY-1 Activity

The effects of selective enzymes on the antimicrobial activity of antibacterial substance JY-1 were shown in Figure 1. The antibacterial activity was not affected by treatments with neutral proteinase and proteinase K. It was decreased partially by trypsin and pepsin treatment. The effects of heat and pH treatment on the antimicrobial activity of the substance were shown in Figure 2. The remaining antibacterial activity gradually decreased with increasing treatment temperature. The antibacterial activity was eliminated after autoclaving (121°C for 30 min). The bacteriocin JY-1 was active over a wide pH range between 2 and 10, but the activity was lower in pH 9 and 10.



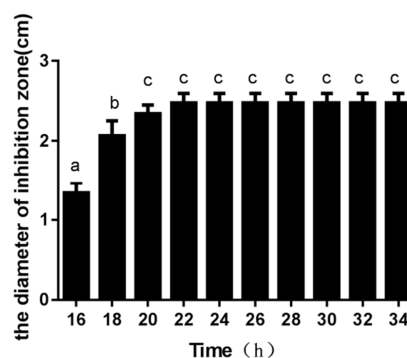
**Figure 1.** Effect of selective enzymes on antimicrobial activity of the bacteriocin-like substance.



**Figure 2.** Effect of heat and pH changes on antimicrobial activity of the bacteriocin-like substance. A. heat treatment; B. pH treatment.

### 3.3. Bacteriocin Production During Growth

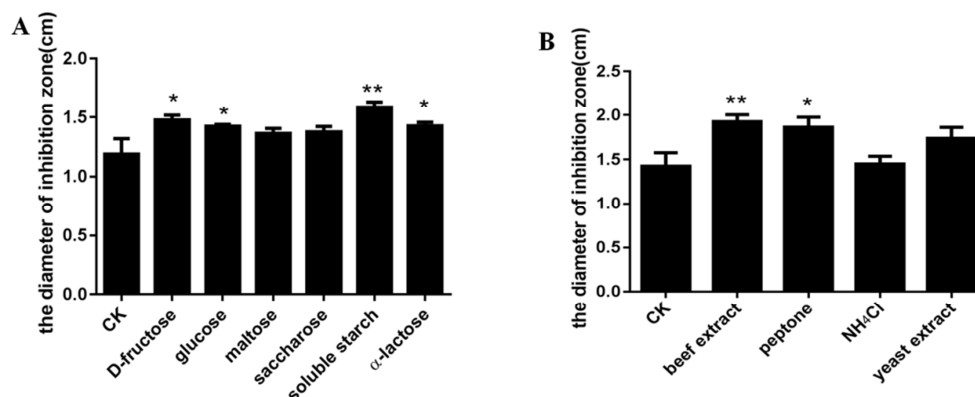
Production of bacteriocin-like substance JY-1 in LB broth was dependent on the bacterial growth phase (Figure 3). During the course of antimicrobial production, the inhibitory activity increased significantly according to cultivation time up to 22 h. Maximum antimicrobial activity was observed at fermentation times ranging from 22 to 34 h.



**Figure 3.** Bacteriocin-like substance JY-1 production during the growth of *Bacillus* spp. JY-1 in LB broth at 37°C.

### 3.4. Effect of Carbon and Nitrogen Sources on the Production of Bacteriocin-like Substance in Soy Whey Based Medium

Soy whey is rich in various useful compounds, such as proteins, polysaccharides and polyphenols [17]. Concentrations of carbon and nitrogen sources in the growth media are known to play a key role in amelioration of the overall physiological response of the organism in terms of



**Figure 4.** Effect of supplement of carbon resources or nitrogen resources on the production of bacteriocin-like substance in soy whey. A. Carbon resources; B. nitrogen resources.

## 4. Discussion

Several hundred wild-type forms of *Bacillus* sp. have been studied in terms of the potential to produce numerous antimicrobial substances, and some *Bacillus* spp. have been identified as generally recognized as safe (GRAS). The production of bacteriocins or bacteriocin-like substances has been already reported for many *Bacillus* species.

The present study reports the optimal production of a bacteriocin-like substance from *Bacillus* spp. JY-1, which isolated from the Chinese traditional fermented soybean. Unlike some bacteriocins from *Bacillus* spp. that have a relative narrow inhibitory spectrum [19], the JY-1 supernatant showed a moderate spectrum, including *Listeria*, *Salmonella*, *Shigella*, *Staphylococcus*, *Clostridium* and *Bacillus*, especially *L. monocytogenes*. Similar results were reported for *B. lentus* NG121 [20], *B. amyloliquefaciens* BFP011 [8] and *B. licheniformis* MCC 2016 [21], but differs from *B. amyloliquefaciens* EMD17 [5] and *B. subtilis* KU43 [22], which were not inhibitory to *Salmonella*.

The production profile of bacteriocin-substance of JY-1 was similar to previous study, which reported that most of the *Bacillus* antimicrobial compounds are produced in the late log phase maybe due to sporulation [23].

There was a significant loss of inhibitory activity after treatment with trypsin and pepsin, suggesting that it is proteinaceous in nature. These results indicated that this antimicrobial peptide possible can survive in the intestinal environment. There results were different to bacteriocin or bacteriocin-like substance produced by *B. amyloliquefaciens* ZJHD3-06 or An6, which were sensitive to the proteolytic action of proteinase K [24, 25]. The pH and heat-stable

production of antibacterial substances [18]. Results in Figure 4A revealed that addition of carbon increased the production of bacteriocin JY-1, especially the soluble starch. The growth of strain JY-1 in the presence of peptone or beef extract yielded a higher production of bacteriocin-like substance (Figure 4B). However, the addition of ammonium chloride had no effect on the production of bacteriocin.

characteristics of bacteriocin-like substance JY-1 showed its potential use as a food preservative. The heat and pH stabilities are very useful characteristics in the application of bacteriocin as food preservatives, because many food processing procedures involve a heating step and/or acidic or alkaline environment [26].

Soy whey from tofu production still contains a substantial amount of nutrients from soymilk, consisting of close to 1% of carbohydrates (predominantly stachyose and sucrose), 0.1-0.8% of proteins, 0.4-1.0% of fats and approximately 0.4% of minerals [27]. Although it is generally considered a waste product, the high-nutrient content of soy whey could enable its value-added use as a substrate for microbial fermentation and production of materials and products. In the study conducted by Mitra *et al.* [13] demonstrated that high-yield of nisin was produced by the successful use of soy whey for fermentation of *Lactococcus lactis*. In present study, we demonstrated that soy whey was suitable for growth of *Bacillus* spp. JY-1, and antimicrobial activity was observed in the supernatant. Supplement of carbon resources stimulated the growth of JY-1 and production of bacteriocin-like substance.

Especially, a high-yield of the bacteriocin-like substance was observed with supplement of soluble starch in soy whey. The microorganisms reported to be active producers of amylolytic enzymes capable of digesting raw starch have mostly been fungi and few bacteria. Earlier studies have reported alpha amylase production by *Bacillus* sp. [28], *B. amyloliquefaciens* [29], *B. licheniformis* [30] and *B. subtilis* [31].

In present study, supplement of soluble starch in soy whey enhanced the production of bacteriocin-like substance, which indicates that *B. spp* JY-1 can secrete amylase. Yeast extract

contains more growth factors and has relatively larger portion of free amino acids and short peptides of 2 or 3 amino acids than protein hydrolysates [32]. Results obtained in present study indicated that the ability to efficiently produce bacteriocin-like substance using value-added agricultural waste materials such as soy whey may facilitate wider use of this once cost-restrictive antimicrobial. Further studies were needed to purified the bacteriocin-like substance.

## 5. Conclusion

The properties of bacteriocin-like substance produced by *B. spp.* JY-1 was determined. Results obtained indicated that bacteriocin-like substance exhibited antimicrobial activity to most tested bacteria, including *Listeria*, *Salmonella*, *Shigella*, *Staphylococcus*, *Clostridium* and *Bacillus*. The production of bacteriocin-like substance by JY-1 reached at maximum levels at the stationary phase. In addition, bacteriocin HY07 is heat-resistant and stable between pH 2 and 10. *B. spp.* JY-1. The production of bacteriocin-like substance produced by JY-1 in soy whey was observed in present study. Supplement of soluble starch or beef extract in soy whey yielded a higher production of bacteriocin-like substance. The results obtained makes this bacteriocin-like substance as antimicrobial agents for control of pathogenic and spoilage microorganisms.

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