Optimization of Conditions for the Maximum Bacteriocin Production of *Enterococcus faecium* DB1 Using Response Surface Methodology

Hye Young Choi, Joon Soo Kim, and Wang June Kim*

Department of Food Science and Technology, Dongguk University, Seoul 100-715, Korea

Abstract

The bacteriocin-producing lactic acid bacteria *Enterococcus faecium* DB1 was isolated from Korean traditional *gajami sikhae*. Culture conditions were optimized by response surface methodology (RSM) to maximize bacteriocin DB1 production. *E. faecium* DB1 displayed the highest bacteriocin activity when grown in modified MRS medium containing sucrose, rather than glucose, as a carbon source. The effects of temperature, initial pH, and sucrose concentration were tested to determine the optimum conditions for maximum bacteriocin production by *E. faecium* DB1. A central composite design was used to control the three variables in the experiment. RSM revealed that the optimum values for bacteriocin production were 27.66 g/L sucrose, temperature of 34.37°C, and an initial pH of 6.54. A 2.08-fold increase in bacteriocin production was obtained with sucrose-containing MRS medium compared to production in standard MRS medium.

Key words: bacteriocin, *Enterococcus faecium*, *gajami sikhae*, optimization, response surface methodology

Introduction

In recent years, bacteriocins are potentially useful as natural replacements for synthetic food preservatives against antibiotic resistant pathogens and spoilage bacteria. Bacteriocins are ribosomally synthesized, hydrophobic or amphiphilic peptides of bacterial origin. Although bacteriocins may be produced by Gram-positive and Gram-negative bacteria, those from lactic acid bacteria (LAB) are of particular interest due to their potential use in the food industry as natural safe food preservatives (Cleveland *et al.*, 2001; Kim, 1993; O'Sullivan *et al.*, 2002). They have relatively narrow antimicrobial spectra as they are only lethal to bacteria closely related to the producer strain, are digested by protease (Ross *et al.*, 1999; Senesi *et al.*, 2001).

Bacteriocins act on target cell membranes resulting pore formation, leads to a rapid efflux of small cytoplasmic molecules, ions from the target cells and the collapse of the proton motive force (PMF), leading to the cell death (Jack *et al.*, 1995). Due to the different modes of antibacterial action, these antibacterial peptides (ABP) circumvent the problems related to the development of antibiotic resistance.

As reported by Cotter *et al.* (2005), "bacteriocins can be used to confer a rudimentary form of innate immunity to foodstuffs", for this reason, production of bacteriocins by LAB and non-LAB strains, as well as their application to the biopreservation of fermented and non-fermented foods have been investigated extensively (Settanni and Corsetti, 2008).

Bacteria of the genus *Enterococcus* are widespread in various traditional food systems, especially those from animal origin, such as dairy products (Foulquie' Moreno *et al.*, 2006). This genus can become an important part of the fermented dairy microflora and its source seems to be not necessarily related with faecal contamination (Giraffa, 2003). Bacteriocins produced by enterococci have gained interest because many species of enterococci are capable of producing a variety of enterocins with activity against *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium* spp. (Floriano *et al.*, 1998; Franz *et al.*, 1999; Gelsomino *et al.*, 2001). Therefore, the use of enterocins or the enterocin-producing starter culture or co-culture, during food fermentation has received special attention as an excellent preservation method to control emergent pathogenic bacteria (Giraffa *et al.*, 1995; Sabia *et al.*, 2002).

Bacteriocin production can be influenced by culture conditions, among them are medium composition, incubation atmosphere, pH, temperature, and microorganism growth phase (Gänzle *et al.*, 1999). Therefore, the optimization of environmental conditions is very important for the enhance-

*Corresponding author: Wang June Kim, Department of Food Science and Technology, Dongguk University, Seoul 100-715, Korea. Tel: 82-2-2260-3373, Fax: 82-2-2260-3373, E-mail: wjkim@dongguk.edu

ment of bacteriocin production. The effects of pH and temperature are very important for bacteriocin production and have been studied by several LAB such as *Lactococcus lactis* (De Vuyst and Vandamme, 1995), *Lactobacillus casei* (Vignolo *et al.*, 1995) and *Leuconostoc mensenteroides* (Krier *et al.*, 1998). Optimization of medium by the classic method involves changing one independent variable while fixing all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables (Adimarayana *et al.*, 2003) and also may result in wrong conclusions (Oh *et al.*, 1995). Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum condition of factors for desirable responses (Li *et al.*, 2001). This method was successfully applied in many areas of biotechnology, including some recent studies on bacteriocin production (Leal-Sánchez *et al.*, 2002; Li *et al.*, 2001).

In our previous paper, we have reported that *Enterococcus faecium* DB1, isolated from Korean traditional *gajami shikae*, produced a narrow spectrum bacteriocin DB1 that inhibits the growth of *Listeria monocytogenes*, *Lactobacillus curvatus* and *Pediococcus acidilactici*. Inactivation of antimicrobial activity was observed after treatment with proteolytic enzymes and α -amylase, and it was heat stable, 16.5 kDa in size. Maximum activity (1,280 AU/mL) against *L. monocytogenes* was observed in MRS broth (Lee and Kim, 2010).

The purpose of this study was to evaluate the effect of three variables including temperature, initial pH, and sucrose concentration in bacteriocin production and to optimize culture condition for production of bacteriocin by *Enterococcus faecium* DB1 through response surface methodology (RSM).

Materials and Methods

Bacterial strains and media

The bacteriocin DB1-producing strain used in this study was *Enterococcus faecium* DB1, previously isolated from *gajami shikae*. *L. monocytogenes* ATCC 19111 was used as the indicator strain for the bacteriocin activity assay. All tested microorganisms were maintained in glycerol stock (20%) at -80°C and activated twice in appropriate broth before use. *Enterococcus faecium* DB1 was cultured in MRS medium (Difco, USA). *L. monocytogenes* was cultured in brain heart infusion (Difco, USA) at 37°C .

Bacteriocin DB1 assay

Antimicrobial activity of *E. faecium* DB1 was investigated by agar well diffusion assay. In an agar well diffusion assay, *E. faecium* DB1 was grown in MRS broth for 16 h at 37°C , and the supernatant was obtained by centrifugation (Sorvall RC-5C Plus, Dupont Co., USA) at 6,000 g for 10 min at 4°C . The pH of supernatant was adjusted to 6.5 by adding 5 N NaOH, and filtered through 0.22 μm -pore-size cellulose acetate filter (Advantec Dismic-13CP, Toyo-Rochi, Japan). Each aliquot of 100 μL of cell-free supernatant was placed into wells (diameter of 9 mm) of the agar plate pre-inoculated with indicator strains ($\leq 10^7$ CFU/g), and the plates were kept at 4°C for 4 h to allow for diffusion before incubation. After 24 h of incubation, the plates were observed for the presence of inhibition zone. The bacteriocin titer was determined by the serial 2-fold dilution method against *L. monocytogenes* ATCC 19111. The bacteriocin titer was defined as arbitrary units (AU/mL). One AU was defined as the reciprocal of the highest dilution at which the growth inhibition was detectable.

Selection of the best carbon source for bacteriocin production

The MRS broth contains peptone, beef extract, yeast extract, glucose, polysorbate 80, ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, and dipotassium phosphate. For selection of the best carbon source, some carbon sources were tested individually: glucose, lactose, mannose, fructose, maltose, sucrose, and sorbitol. Each carbon source was added at 2%(w/v) of the basal MRS medium, replacing the 2% glucose. After inoculation of *E. faecium* DB1 (1% of inoculum), incubation was performed at 37°C . During the 27 h of growth cycle, samples were taken at every 2 h intervals and measured pH, and optical density (OD) under 600 nm wavelength using spectrophotometer (Smart Plus SP-1900PC, Woongki Science, South Korea). The antimicrobial activity was also measured at every 2 h by using agar well diffusion test. Ten mL of cultures were aseptically taken and centrifuged (10,000 g, 15 min at 4°C). The pH of supernatant was adjusted to 6.5 and filtered through 0.22 μm -pore size cellulose acetate filter. Cell-free supernatant was assayed using serial 2-fold dilution. Sterile MRS broth was used as diluents. Each diluted cell free supernatant (100 μL) was loaded on well (9 mm) of BHI agar lawn containing *L. monocytogenes* ATCC 19111. The antimicrobial activity of bacteriocin DB1 was expressed as AU/mL. All experiments were performed in duplicate.

Experimental design of RSM

After selection of the best carbon source, the next step was to determine the optimal levels of three variables (temperature, initial pH and sucrose concentration) on bacteriocin production. For this purpose, the response surface approach by using a set of experimental design (central composite design, CCD) was performed. For the three factors, this design was made up of a full 2^3 factorial design with its eight points augmented with six replications of the center points (all factors at level 0) and the six star points, that is, points having for one factor an axial distance to the center of $\pm \alpha$, whereas the other two factors are at level 0. In most cases the full 2^n factorial design is used in the cube portion in a CCD and thus the α value is calculated with the following formula (Ruguo, 1999):

$$\alpha = \frac{[(\sqrt{2^n + 2 \times n + r - 2^{n/2}})^2 \times 2^n]^{0.25}}{\sqrt{2}}$$

In this study, the axial distance α was chosen to be 1.68 to make this design orthogonal. The central composite design was proceeded to obtain a quadratic model, consisting of trials plus a star configuration to estimate quadratic effects and central points to estimate the pure process variability and reassess gross curvature, with active substances production as response. The CCD used was generated by “Minitab” software (version 15). According to this design, 20 experiments were conducted containing six replications at the center point for estimating the purely experimental uncertainty variance. In this study, three key variables with three concentration levels were adopted. To test the influence of sucrose concentration, sucrose was added as 9, 20, and 31 g/L. To test the effect of the initial pH on bacteriocin production, MRS media were adjusted to different pH values (pH 5.5, 6.5, 7.5) with 5 N NaOH or 5 N HCl. To test the effect of the incubation temperature on bacteriocin production, modified MRS media were adjusted to different temperature (30, 37, and 44°C).

And each variable was designated as -1, 0, and +1, respectively, given in Table 2. The central values (0 level) chosen for experimental design were: temperature, 37°C; initial pH, 6.5 and sucrose concentration, 20 g/L. In developing the regression equation, the test factors were coded according to the following equation:

$$xi = \frac{Xi - Xo}{\Delta Xi}$$

Where xi is the coded value of the i th independent variable, Xi the natural value of the i th independent variable, X_0 the natural value of the i th independent variable at the

Table 1. Growth and bacteriocin production of *E. faecium* DB1 with different carbon sources

Modified MRS media	Growth (OD ₆₀₀)	Bacteriocin activity (AU/mL) ¹⁾
Not added	0.850	160
Glucose	3.065	1280
Lactose	2.870	1280
Mannose	2.685	1280
Fructose	1.975	640
Maltose	3.175	1280
Sucrose	3.245	2560
Sorbitol	0.935	320

¹⁾AU/mL represents the reciprocal of the highest inhibitory dilution in the two-fold dilution assay of bacteriocin.

Table 2. Coded and real values of variables in fractional factorial design

Variables	Level of variables		
	-1	0	1
X ₁ : Sucrose (g/L)	9	20	31
X ₂ : Temperature (°C)	30	37	44
X ₃ : Initial pH	5.5	6.5	7.5

center point, and ΔXi the step change value (ΔXi is 7 for temperature, 1 for initial pH, and 11 for sucrose concentration). The levels of these three variables were selected to approach the region where high bacteriocin activity was expected. If the difference was significant between the mean of the center points and that of the factorial points ($p < 0.05$), the optimum would be near or within the experimental design space. Otherwise the optimum would be outside the experimental design space and the method of steepest ascent should be applied (Xiaojie *et al.*, 2003). The central composition design of four variables in coded along with titer as responses is presented in Table 3.

Results and Discussion

Selection of the best carbon source for bacteriocin production

The effect of the carbon source on cell growth and bacteriocin production was determined using MRS medium supplemented with 2% of different carbon sources in place of glucose. As shown in Fig. 1, the highest growth was observed in medium containing sucrose; maltose, glucose and lactose were also suitable carbon sources for growth. Among the media tested, the *E. faecium* DB1 produced maximum bacteriocin in sucrose (2,560 AU/mL) (Fig. 2).

Table 3. Experimental design and results of central composite design for optimization of bacteriocin production

Run	Variables			Bacteriocin production (AU/mL)
	X ₁ Sucrose(g/L)	X ₂ Temperature (°C)	X ₃ Initial pH	
1	-1	-1	1	1280
2*	1.682	0	0	2560
3	1	1	1	320
4c	0	0	0	1280
5c	0	0	0	1280
6	-1	-1	-1	1280
7*	0	-1.682	0	1280
8c	0	0	0	2560
9c	0	0	0	2560
10	1	-1	1	1280
11*	0	0	1.682	640
12*	-1.682	0	0	1280
13	-1	1	1	640
14	-1	1	-1	640
15c	0	0	0	2560
16*	0	0	-1.682	80
17	1	1	-1	640
18	1	-1	-1	1280
19c	0	0	0	2560
20*	0	1.682	0	80

c, center point; *, axial point

Fig. 1. Effect of carbon sources on cell growth (□) and bacteriocin DB1 production (■) in *E. faecium* DB1.

Several authors reported that higher bacteriocin activities were observed with increased carbon concentrations, thus our results showing lower bacteriocin recovery with reduced carbon source are not surprising. Although *E. faecium* DB1 grew without carbon source, there was no bacteriocin production (Fig. 1). This growth may be derived from the utilization of residual sugar or another nutrient, such as yeast extract or peptone, present in MRS medium in sufficient

Fig. 2. Antimicrobial activity of bacteriocin DB1 in modified MRS medium containing sucrose against *L. monocytogenes* by agar well diffusion assay.

quantities to allow *E. faecium* growth but insufficient for normal synthesis of bacteriocin. Bacteriocin activities do not always correlate with cell concentration or growth rate of the producer (Bogovic-Matijasic and Rogelj, 1998). Higher levels of bacteriocin production are often given at conditions that differ from those required for optimal growth (Aesen *et al.*, 2000; Krier *et al.*, 1998; Parente and Ricciardi, 1994).

The soil environment contains a variety of carbohydrates derived from plant, animal and microbial material that are available to heterotrophic bacteria as carbon source. The bacterial species using these carbohydrates produce a range of enzymes to facilitate this process, some of which are extracellular and are exported into the environment to break down the carbohydrates into smaller entities that can be taken up by the bacterium and metabolized.

Glucose is considered the main carbon source by all microorganisms due to its size, rapid uptake, utilization and cellular energy conversion. However, some bacteria have a complete enzymatic machine that allows them to use complex carbohydrates; for example, *E. faecium* shows a variable sucrose fermentation pattern (Barnes, 1964).

Sucrose is composed of a glucose unit linked to a fructose unit via a glycosidic linkage, and it is the most abundant disaccharide in the environment because of its origin in higher plant tissues. Three main classes of enzymes have been implicated in the formation and cleavage of glycoside linkages, including the catabolic enzymes, the sucrose hydrolases and phosphorylases (Kitaoka and Hayashi, 2002), and the biosynthetic glycosyl nucleotide glycosyltransferases, which are responsible for the synthesis of carbohydrate polymers produced by bacteria (Steinmetz, 1993).

Fig. 3. 2D contour plot and 3D response surface curve. A, temperature and sucrose concentration; B, initial pH and sucrose concentration; C, initial pH and temperature

A number of different enzymes, belonging to either the hydrolytic or the phosphorolytic class, catalyze the catabolism of intracellular sucrose. These enzymes catalyze the hydrolysis of terminal fructose moieties from fructose-containing disaccharides, trisaccharides and polymers, with varying specificity. The most well-characterised enzymes within this group are sucrose-6-phosphate (P) hydrolases which cleave sucrose-6-P to yield glucose-6-P and fructose, and which are usually associated with the phosphoenolpyruvate-dependent phosphotransferase system (PTS). The fructose resulting from the catabolism of either sucrose or sucrose-6-P is generally phosphorylated by ATP-dependent fructokinases to give fructose-6-P, an intermediate in the glycolytic pathway. The glucose-6-P generated by the sucrose-6-P hydrolases directly is incorporated into the glycolytic pathway.

Type and concentration of carbon sources reported are the most influent factors in bacteriocin production (Delgado *et al.*, 2007). Several authors reported that glucose and sucrose were reported to be suitable carbon sources for nisin Z production (Matsusaki *et al.*, 1996), lactose for nisin-like bacteriocin production (Cheigh *et al.*, 2002), glucose for Streptococcin AFF22 production (John and Ingrid, 1991), nisin production is affected by carbon source regulation, nitrogen sources and phosphorus (De Vuyst and Vacdamme, 1992; De Vuyst and Vacdamme, 1993). However our results showed that only sucrose was more suitable carbon source for bacteriocin DB1 production than glucose or lactose. Therefore, more study is needed to reveal the

correct mechanism of sucrose as a carbon source.

Response surface methodology

The results of central composite design experiments for studying the effects of three variables, sucrose concentration, temperature, and initial pH, on bacteriocin production are presented in Table 3. The corresponding response surface is shown in Fig. 3A, 3B, and 3C, where a point for maximal bacteriocin production can be found. Fig. 3A shows 2D contour plot and 3D response surface curve for variation in the yields of bacteriocin. From the response surface plots, it is easy and convenient to understand the interactions between two variables. The optimum values of the tested variables for the production of bacteriocin were 27.66 g/L sucrose, temperature 34.37°C and initial pH 6.54. In comparison to the production of MRS medium, 2.08-fold increase had been obtained.

The effects of pH and temperature are very important for bacteriocin production and have been reported for several bacteriocins produced by *E. faecium* (Parente and Ricciardi, 1994), *L. casei* (Vignolo *et al.*, 1995) and *L. mesenteroides* (Krier *et al.*, 1998). Using the RSM methodology, we demonstrate that these parameters are also fundamental for bacteriocin production by *E. faecium* DB1 as well. Decrease in bacteriocin productivity at high sucrose concentration could be explained by a relatively high lactic acid concentration, which might inhibit cell growth and bacteriocin production.

The traditional “one-factor-at-a-time approach” is an operation frequently used in medium optimization to obtain

high yields of the desired metabolic products in a microbial system. But that method disregards the complex interactions among various physicochemical parameters (Abdel-Fattah *et al.*, 2005). Statistically based experimental designs such as factorial design and response surface analysis particularly fulfills this requirement. RSM can be used to study the effects of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments (Elibol, 2004; Piyushkumar *et al.*, 2007).

RSM has been a popular and effective method to solve multivariate problems and optimize responses in many types of experimentation (Dale *et al.*, 2007; Park *et al.*, 2007). The method of RSM for improvement of fermentation was described in detail by Strobel and Sullivan (Strobel and Sullivan, 1999). As large amounts of bacteriocin are necessary to test their preservative efficiency in natural environments, establishment of the factors and levels influencing maximal production would lead to a more effective recovery of these antimicrobial compounds from a defined laboratory culture medium (Aymerich *et al.*, 2000; Daeschel and Hoover, 1993).

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