

## Release Profile and Antimicrobial Activity of Nisin Control-released from Agar Gel Foods

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### Abstract

The release profiles of nisin from nisin-incorporating gel foods and the antimicrobial activities of the gels on the growth of *Brochothrix thermosphacta* in an aqueous system containing the gels have been investigated. A linear regression model was applied to determine the diffusion coefficient ( $D$ ) for the diffusion of nisin in the gel. The antimicrobial activities of nisin released from 1, 2 and 3% (w/v) agar gels on the growth of *B. thermosphacta* in a broth medium with and without nisin were investigated. The  $D$  decreased from  $1.2 \times 10^{-2}$  to  $8.2 \times 10^{-3}$  and  $6.4 \times 10^{-3}$   $\text{cm}^2/\text{s}$  as the agar concentration in the gel increased from 1 to 2 and 3% (w/v), demonstrating the diffusion rate in the gels can be controlled by the agar concentration in gel. The agar gel incorporating nisin inhibited the *B. thermosphacta* growth in the broth medium by prolonging the lag phase. The growth inhibition was enhanced by the addition of nisin in the medium. The results of this study exhibited that the gel food is a feasible nisin delivery system with a controlled release achieved by the adjustment of agar concentration in the system, demonstrating the potential of nisin-incorporating gel for preserving particulate-containing drinks.

**Key words:** nisin, diffusion coefficient, agar gel, *Brochothrix thermosphacta*

### Introduction

Direct surface application of antimicrobial substances onto food products, including dipping and dissolving of antimicrobials, exhibits limited antimicrobial effectiveness since the active substances are neutralized on contact or diffuse rapidly from the surface into the products. Antimicrobial incorporation in the food matrix has advantages over the direct surface applications because the food system can be designed to slow the diffusion of the antimicrobial agent (Min & Krochta, 2005). By slowing the diffusion to the food surface, the substance activity at the surface of food is maintained. Thus, smaller amount of antimicrobials is needed in the antimicrobial-incorporating food material to achieve a target shelf life as compared to the food material prepared with direct surface application of antimicrobials. The diffusion of the incorporated antimicrobials can be controlled by controlling food compositions (Han, 2000).

Nisin, a bacteriocin produced by *Lactococcus lactis* spp., can inhibit Gram-positive foodborne pathogens and spoilage microorganisms (Delves-Broughton et al., 1996; Park et al., 2009), including *Brochothrix thermosphacta* which is frequently involved in the spoilage of meat products stored aerobically or vacuum packaged at refrigerated conditions (Cayré et al., 2005; Kilcher et al., 2010). Nisin is applied as a preservative and an antimicrobial agent to a variety of foods, including meat, fish, dairy, beverage, and salad dressing products (Sobrino-López & Martín-Belloso, 2008; Ercolini et al., 2010; Abdollahzadeh et al., 2014; Gharsallaoui et al., 2016). Antimicrobial incorporation in food systems results in the preservation of foods by prolonging the lag phase of spoilage microorganisms (Sung et al., 2013). To extend the effect of antimicrobial application, antimicrobial has been embedded in polymer networks of food packages (e.g., films and coatings) to slow down its release to food systems. The application of the biodegradable polylactic acid (PLA) film containing nisin reduced the numbers of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enteritidis* in culture media and liquid foods by 2.5-4.5 log cycles (Jin & Zhang, 2008). The effectiveness of nisin-incorporating edible films on food preservation was also reported previously (Sebti et al., 2004; Resa et al., 2014;

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Received December 16, 2016; revised January 25, 2017; accepted January 26, 2017

Krivorotova et al., 2016). However, these experiments have studied the antimicrobial activity of nisin only in polymeric films. No previously-published research papers have reported the release characteristics of nisin in a gel food system and the release-dependent antimicrobial effects of nisin in the gel food system. Thus, the objectives of this study were to determine the diffusion profile of nisin in agar gels, model gel foods, with determination of the diffusion coefficient for the diffusion of nisin in the gels, and investigate antimicrobial effects of nisin-incorporating agar gels at different concentrations of agar on the growth of *B. thermosphacta* in a microbial growth media.

## Materials and Methods

### Bacterial strain and culture conditions

*B. thermosphacta* was selected as the target microorganism to determine the antimicrobial activity of nisin released from the agar gel. All purpose Tween (APT, Difco, Becton Dickinson, Sparks, MD, USA) agar slants were used to maintain bacterial culture at 4°C. *B. thermosphacta* (ATCC 11509) was cultured in brain heart infusion (BHI, Difco, Detroit, MI, USA) at 25°C. Cells from 48 h-culture were collected by centrifugation (4,000×g, 15 min, 22°C; GyroSpin, Gyrozen Co. Ltd., Seoul, Korea) and suspended in 0.1% (w/v) peptone water. The suspension (approximately 10<sup>9</sup> CFU/mL) was diluted with 0.1% peptone water for inoculation.

### Preparation of nisin-incorporating agar systems

Nisin (Sigma-Aldrich Co., St. Louis, MO, USA) powder (1 g) was dissolved in 10 mL distilled water in a beaker with a stirring bar. The nisin solution was covered and agitated overnight to make nisin dissolve thoroughly. After 24 h, agar powder (A-1296, Sigma-Aldrich Co.) were dissolved in distilled water to prepare 1, 2, or 3% (w/v) agar solutions. Agar solutions were autoclaved at 121°C for 15 min and left in a water bath at 50°C. After equilibrium, the nisin solution was slowly transferred to the agar solution. The nisin-agar solution was left overnight at 24±2°C in the dark to form agar gel model foods. The concentration of nisin in the gel was 2% (w/v) and those of agar were varied at 1, 2 and 3% (w/v). The gel thickness was 1 cm.

### Determination of nisin release profile

The scheme of diffusion experiment is illustrated in Fig. 1. Agar gel model food was trimmed into cylinders of 1.0 cm diameter by 3.0 cm length using a sterile cheese borer. Each gel without edge damage was carefully transferred to a

volumetric beaker, containing 100 mL distilled water. The beakers were covered with paraffin films and placed on a shaker (Julabo SW22, Labortechnik GmbH, Seelbach, Germany) rotating at 60 rpm at 24±2°C. The absorbance was measured at 280 nm every 1 h using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Piscataway, NJ, USA) (Breukink et al., 1998). The concentration of nisin released into water was calculated from a nisin standard curve. The release rate of nisin was determined with the ratio of the level of nisin released to the initial level of nisin in the agar.

The diffusion through the film was determined from the data obtained using the following solution for diffusion in a plane sheet, derived from Fick's law (Crank, 1975). In this experiment, agar gel was assumed to be a homogeneous system containing nisin molecules uniformly in the gel matrix.

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} \exp(-D(2n+1)^2\pi^2t/4L^2) \quad (1)$$

where,  $M_t$  = mass of nisin released from the gel at time  $t$ ;  $M_\infty$  = mass of nisin released from the gel at infinite time;  $n$  = natural numbers;  $D$  = diffusion coefficient;  $L$  = half thickness of agar gel.

Eq. (1) can be simplified to Eq. (2) by a log transformation using only the first term in the summation series (Crank, 1975):

$$\ln\left(1 - \frac{M_t}{M_\infty}\right) = \ln\frac{8}{\pi^2} - \frac{D_c\pi^2t}{4L^2} \quad (2)$$

When only the experimental data over 40% of the maximum release were used, the following linear regression can be used:

$$Y_i = b_0 + b_1X_i + e_i \quad (3)$$

where  $Y_i = \ln(1 - M_t/M_\infty)$ ;  $X_i$  = time;  $\beta_0$  = intercept ( $\ln 8/\pi^2$ );  $\beta_1$  = slope ( $-D\pi^2/4L^2$ );  $e_i$  = error.

Diffusion coefficient ( $D$ ) can be determined as follows (Han et al., 2000):

$$D = -\text{slope}/(\pi^2/4L^2) \quad (4)$$

### *B. thermosphacta* inhibition study

Experiment was conducted with the set-up illustrated in Fig. 1 using the *B. thermosphacta*-inoculated APT broth instead of distilled water. The inoculum of *B. thermosphacta* at 8 log CFU/mL ( $OD_{600} = 0.915$ ) was transferred to 100 mL APT broth. Three experiments were conducted. First, the inhibitory effect of nisin in APT broth on the growth of *B. thermosphacta*

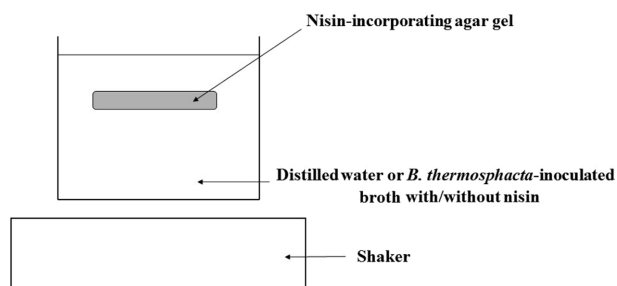


Fig. 1. Scheme of nisin release experiments.

in the broth was investigated. The concentrations of nisin in the broth were 0.1 and 0.5% (w/v). Secondly, the antimicrobial activities of the agar gel cylinders incorporating nisin (2%, w/v) were evaluated in nisin free-APT broth. The concentrations of agar in the gels were 1, 2 and 3% (w/v). Lastly, the inhibitory effects of nisin released from agar gels with different concentrations of agar were studied in APT broth containing nisin at 0.1%. The cell density of the APT broth was measured at 600 nm. The measurement was made every hour with the samples in the shaker rotating at 60 rpm during incubation at  $24 \pm 2^\circ\text{C}$  for 120 h.

### Statistical analysis

All experiments were replicated in two times. Each observation within each replication was determined in triplicate. The slope of the data points for  $D$  determination was determined by fitting a straight line to the data using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

## Results and Discussion

### Effects of agar concentration on the nisin diffusion

The highest release of nisin was shown in 1% agar gel, followed by 2% and 3%. The maximum release of nisin, demonstrated with 1% agar gel was 86%, while for 2% and 3% agar gels, the levels of release were 77% and 67%, respectively (Fig. 2). After about 30 h of release, nisin reached a steady state with its maximum release. At its steady-state, microorganisms could be continuously attacked by nisin which releases at maximum.

The experimental data which are over 40% of the maximum release rate were fit by the linear regression model of Eq. (3) (Fig. 3). Fig. 3 shows that the steady-state of the release has a very good fit to the linear model. The high linearity of  $\ln(1 - M_t/M_\infty)$  to time (Fig. 3) indicates that this linear regression model is adequate to describe nisin release profile from the agar gels. The parameters of the regression models,

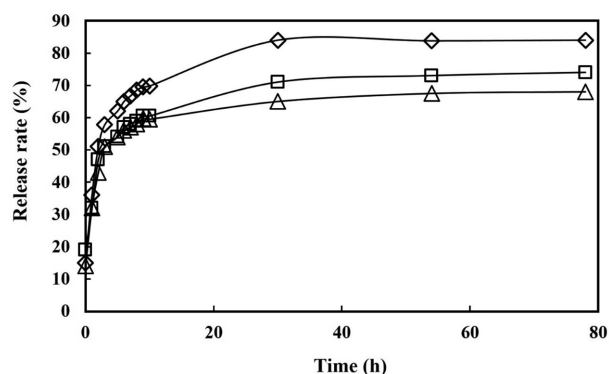


Fig. 2. The release profiles of nisin incorporated in the gel systems of agar at 1, 2 and 3%. Diamond ( $\diamond$ ), square ( $\square$ ) and triangle ( $\triangle$ ) are the absorbance values of the gels containing agar at 1, 2 and 3%, respectively. The concentration of nisin in the gels was 2% (w/v).

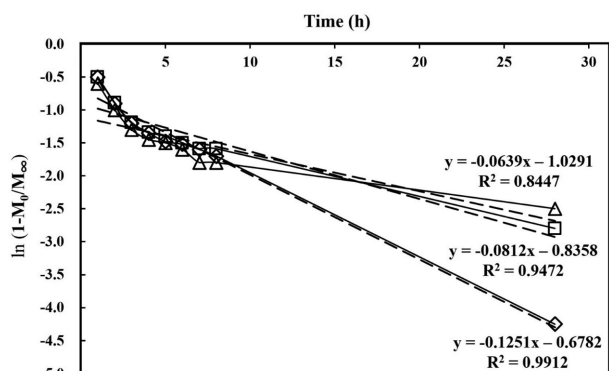


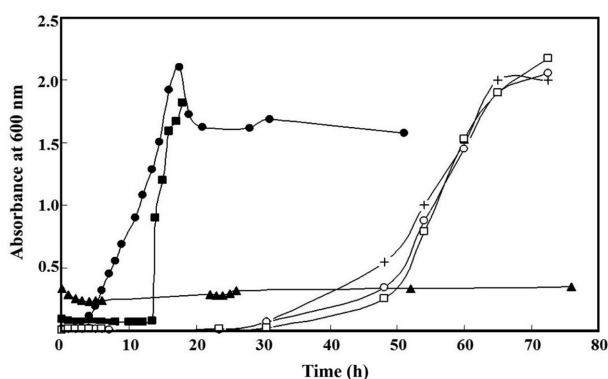
Fig. 3. The release profiles of nisin in the gel samples incorporating nisin (2%, w/v) during storage at  $24^\circ\text{C}$ . The concentration of nisin in the gels was 2% (w/v). Experimental data and model predicted data are shown with symbols and a solid line, respectively. Diamond ( $\diamond$ ), square ( $\square$ ) and triangle ( $\triangle$ ) represent the gel samples of agar at 1, 2 and 3%, respectively.

determined from the release profiles of the gels of 1, 2 and 3% agar, are summarized in Table 1. The results in Table 1 and Fig. 3 demonstrate that the diffusion efficacy of nisin decreases with the increase of agar concentration in the gel. This might be due to formation of a gel network that could hold more nisin at a higher agar concentration. Hence, nisin release rate from agar gels can be controlled by adjusting the agar concentration.

The  $D$  values decreased as the agar concentration increased, agreeing with the release results described previously. The difference between the  $D$  values obtained from 1 and 2% agar gels (0.0044) was higher than the difference between the values obtained from 2 and 3% agar gels (0.0018). No experiments were conducted with agar at the concentration  $>3\%$  since solidification of agar gels became difficult to

**Table 1. Linear regression models and diffusion parameters for the diffusion of nisin in the gel systems containing agar at 1, 2 and 3% (w/v)**

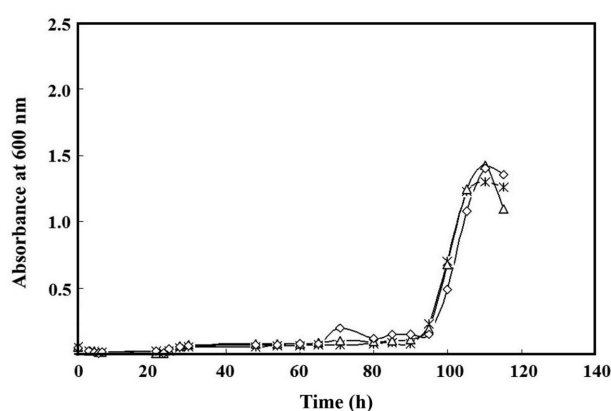
Concentration of agar gels	Linear regression equation	R <sup>2</sup>	Maximum release	Slope	Diffusion coefficient (D, cm <sup>2</sup> /s)
1%	Y=-0.1251X-0.6782	0.997	86.0	-0.1251	1.2×10 <sup>-2</sup>
2%	Y=-0.0812X-0.8358	0.947	77.1	-0.0812	8.2×10 <sup>-3</sup>
3%	Y=-0.0446X-0.8079	0.845	71.9	-0.0639	6.4×10 <sup>-3</sup>



**Fig. 4. Growth of *B. thermosphacta* in APT broth at 24°C.** Closed circles (●) represent cultures grown in the absence of nisin (control) while closed squares (■) and closed triangles (▲) represent cultures grown in the presence of nisin at 0.1 and 0.5 % (w/v), respectively. Open squares (□), open circles (○) and cross symbols (+) represent cultures grown in the presence of the nisin (2%, w/v)-incorporating gels of agar at 1, 2 and 3% (w/v), respectively.

control and gel cylinders were easy to break, which is consistent with the report by Sebti et al. (2004).

Ripoche et al. (2006) investigated nisin diffusion in agar gels at 10°C for 6 days and reported the  $D$  values for the diffusion in the gels containing agar at 3.2, 3.9, and 6.7% as  $4.2 \times 10^{-7}$ ,  $3.4 \times 10^{-7}$ , and  $2.5 \times 10^{-7}$  cm<sup>2</sup>/s, respectively. The  $D$  for nisin diffusion in hydroxypropyl methylcellulose polymer film at 25°C ranged from  $6.5 \times 10^{-7}$  cm<sup>2</sup>/s to  $3.3 \times 10^{-6}$  cm<sup>2</sup>/s in a 3%-agar gel containing fat at 5 or 30% (w/w) (Chollet et al., 2009). In the current study, the  $D$  values were  $1.2 \times 10^{-2}$ ,  $8.2 \times 10^{-3}$ , and  $6.4 \times 10^{-3}$  cm<sup>2</sup>/s with the gels containing agar at 1, 2 and 3%, respectively, and they are relatively high. The inconsistency of the  $D$  values might be due to various factors, including temperature, polymer morphologies including density, crystallinity, tortuosity, degree of crosslinking and branching, and glass transition temperature, and molecular interactions of the diffusants and host polymers (Bawa et al., 1985; Helmroth et al., 2002). The results suggest that the gel food system can be used as a nisin carrier for a controlled release of nisin, which is determined by the concentration of agar in the system.



**Fig. 5. Growth of *B. thermosphacta* at 24°C in APT broth in which nisin was dissolved and the agar gel containing nisin (2%, w/v) was placed.** The symbols, \*,  $\Delta$  and  $\diamond$  represent cultures exposed simultaneously to the nisin-dissolved broth and the nisin-incorporating gel containing agar at 1, 2 and 3%, respectively.

#### Inhibition of *B. thermosphacta* by nisin released from agar gels

Addition of nisin at 0.1 and 0.5% in the APT broth retarded the growth of *B. thermosphacta* in the broth for about 9 and >72 h, respectively (Fig. 4). Treatment with the agar gels incorporating nisin also inhibited the growth of *B. thermosphacta* (Fig. 4). Without nisin in liquid broth solution, the release of nisin affected *B. thermosphacta* growth, prolonging the lag phase of *B. thermosphacta* growth by about 26 h (Fig. 4). These results suggest that nisin-incorporating agar gel is capable of inhibiting the growth of *B. thermosphacta*. A longer inhibition of the growth was observed with the gel containing agar at 1% than the gel with 3% agar. This could be due to a higher release of nisin from the gel at a lower concentration of agar in the gels as described previously.

A simultaneous treatment of *B. thermosphacta* with the nisin (0.1%)-dissolved broth and the nisin-incorporating gel effectively inhibited the microbial growth in the medium, showing the initiation of logarithmic phase on about 95 h, irrespective of the concentration of the agar in the gels (Fig. 5). The effect of the concentration of agar in the gel formulation was not demonstrated in this result. This might be due to

swelling of the gels induced by excessive storage of the gels in the liquid medium (115 h), which could destruct the nisin-hold gel networks at any concentration of agar. If the network were more resistant to moisture absorption, the microbial inhibition could be extended since potential uncontrolled outpouring of nisin into the broth, which can use up nisin immediately, could be prevented. The initiation point of the logarithmic phase of *B. thermosphacta* in the simultaneous treatment (90 h) was longer than the summation of the initiation points in the individual treatments of the nisin (0.1%)-dissolved broth (13 h) and the nisin-incorporating agar gel (30 h). The simultaneous treatment demonstrates its potential use as a means to control *B. thermosphacta* in liquid foods containing the gels as particulates.

### Conclusion

The linear regression model is capable and convenient in determining the diffusivity of nisin in the gel systems with different agar concentrations. The *D* values for nisin decreased with agar concentration in the gels, suggesting the ability of the gel holding nisin increases with the concentration of agar. The growth of *B. thermosphacta* in the broth medium was effectively inhibited by placing the nisin-incorporating agar gel in the medium. The antimicrobial effect of the gel could be adjusted by controlling release of nisin by formulating the gel with different concentrations of agar. Furthermore, the addition of nisin the medium noticeably increased the microbial inhibition. The results presented in this study may be useful for designing gel or gel particulate-containing liquid products using nisin as a natural antimicrobial agent.

### Acknowledgement

This work was supported by a sabbatical year research grant from Seoul Women's University (2015).

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