Advance Publication

J. Gen. Appl. Microbiol.
doi 10.2323/jgam.2026.06.004

Short Communication

Bacillus subtilis var. natto as a naturally optimized biotin biosensor

(Received June 2, 2026; Accepted June 22, 2026; J-STAGE Advance publication date: June 29, 2026)

Wakana Suda[1,2], Yoshiaki Ohashi[3], and Nobuaki Kono[1,4,5,*]

[1] Institute for Advanced Biosciences, Keio University, Yamagata, Japan

[2] Department of Bioscience, Faculty of Life Sciences, Tokyo University of Agriculture, Tokyo, Japan

[3] Fermecutes Inc., Yamagata, Japan

[4] Graduate School of Media and Governance, Keio University, Kanagawa, Japan

[5] Tsuruoka Institute for Gastronomic Innovation, Yamagata, Japan

[*] author for correspondence: Nobuaki Kono email: nkono.a3@keio.jp, Graduate School of Media and Governance, Keio University, Fujisawa, Kanagawa, 252-0882, Japan
ORCID: 0000-0001-5960-7956

Summary (120 words)

Bacillus subtilis var. natto is a naturally occurring biotin auxotroph widely used in traditional soybean fermentation. In this study, we evaluated its potential as a microbial biotin biosensor without genetic modification. Using a two-layer agar diffusion assay, growth responses were examined across biotin concentrations ranging from 0.001 to 200 mg/L. Halo diameters increased proportionally with biotin concentrations. The strain exhibited detectable responses over more than five orders of magnitude of biotin concentration without engineering of biotin transport or biosynthetic pathways. These results demonstrate that *B. subtilis* var. natto can serve as a simple, safe, and cost-effective biotin bioassay platform and highlight the utility of naturally evolved auxotrophic microorganisms as biosensors.

Keywords

Bacillus subtilis var. natto, biotin, biosensor

34

35 **Brief running head**: Natural biotin sensor in natto

36

37

38 Biotin (vitamin B7) is an essential cofactor required for carboxylase reactions

39 involved in fatty acid synthesis, amino acid metabolism, and gluconeogenesis in all

40 domains of life (Tong, 2013). Although many bacteria and plants are capable of

41 synthesizing biotin, animals and numerous microorganisms depend on environmental

42 uptake of this vitamin (Lin and Cronan, 2011). Because humans and livestock cannot

43 synthesize biotin and must obtain it through food or supplementation, its quantification

44 is important in food and feed applications. Consequently, sensitive microbial bioassays

45 for biotin quantification have long been used in food, pharmaceutical, and

46 microbiological studies (Livaniou et al., 2000).

47 Recent studies developed engineered bacterial strains for broad-range biotin

48 bioassays (Ikeda et al., 2013; Finkenwirth et al., 2014). In particular, *Escherichia coli*

49 mutants lacking both biotin biosynthesis and high-affinity biotin transport systems

50 enabled highly sensitive detection across an exceptionally wide concentration range

51 spanning 0.0001 to 200 mg/L (Finkenwirth et al., 2014). However, these systems

52 require multiple genetic modifications and specialized strain construction. Furthermore,

53 the use of genetically modified microorganisms can restrict experimental applications

because of biosafety regulations and containment requirements. In addition, combinations of multiple strains are often necessary to cover broad concentration ranges, reducing the simplicity and convenience of these assay systems.

Bacillus subtilis var. natto is a naturally occurring biotin auxotroph widely used in traditional soybean fermentation (Sasaki et al., 2004; Kubo et al., 2011; Kamada et al., 2015) and has a long history of safe use in food production (Wang et al., 2023). Previous studies demonstrated that the growth of natto strains is strongly dependent on environmental biotin concentrations, suggesting that these bacteria possess highly sensitive physiological responses to biotin availability. Therefore, natto strains may serve as naturally optimized microbial biotin sensors without the need for genetic engineering. In addition, the use of a food-grade microorganism may provide practical advantages over genetically modified biosensor strains by reducing biosafety concerns and facilitating broader applications in research, education, and industrial settings.

In this study, we examined the growth response of wild-type *B. subtilis* var. natto to a broad range of biotin concentrations and evaluated its applicability as a microbial biotin biosensor. We demonstrate that the natto strain exhibits robust growth responses over a remarkably wide concentration range without genetic engineering, suggesting that naturally evolved vitamin-scavenging properties of natto strains may be exploited for

simple and versatile biotin bioassays. The use of naturally occurring natto strains not only provides a novel microbial biosensor platform, but also overcomes practical limitations associated with engineered biosensor strains, including biosafety concerns and the complexity of strain construction and maintenance.

B. subtilis var. natto Miyagino strain (BANK12184) was grown overnight in LB medium at 37°C. Cells were harvested, washed three times with 1× minimal medium, and adjusted to an OD600 of 1.0. A two-layer agar diffusion assay was performed. Bottom plates consisted of Spizizen minimal medium (SMM, Anagnostopoulos & Spizizen, 1961) containing 1.5% agarose. The top layer consisted of SMM containing 0.7% agarose, into which bacterial cells were mixed before pouring onto the bottom layer. Sterile paper disks (8 mm diameter; Advantec, Tokyo, Japan) or circular disks prepared from blotting paper were placed at the center of each plate and loaded with 10 μL of biotin solution at concentrations ranging from 0 to 200 mg/L (0, 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 200 mg/L). Plates were incubated at 30°C for 43 h. Growth halos surrounding the disks were scanned and quantified using Fiji (ImageJ version 1.54p; Schindelin et al., 2012). Halo diameters obtained using paper disks were measured from three independent biological replicates and are presented as mean ± SD. Blotting paper was evaluated in a single experiment.

To evaluate the applicability of the natto strain as a microbial biotin biosensor, disk diffusion assays were performed using paper disks containing different concentrations of biotin. To obtain well-defined halo boundaries, a two-layer bioassay plate system was employed. After incubation at 30°C for 42–43 h, clear halo formation was observed around the disks, and halo diameters increased in proportion to biotin concentration (Figure 1A). Notably, the characteristics of halo formation differed between paper disk and blotting paper, indicating that the choice of paper material can influence the detectable range of the assay. For paper disk, halo diameters were measured from three independent biological replicates, whereas blotting paper was evaluated in a single experiment. Linear regression analysis across the entire concentration range yielded coefficients of determination (R^2) of 0.911 and 0.982 for paper disk and blotting paper, respectively. Although halo formation could be detected at concentrations as low as 0.01 mg/L (paper disk) and 0.001 mg/L (blotting paper), the most linear response was observed within the range of 0.01–100 mg/L ($R^2 = 0.996$) for paper disk and 0.001–100 mg/L ($R^2 = 0.979$) for blotting paper (Figure 1B).

Consistent with these observations, wild-type *B. subtilis* var. natto exhibited clear growth responses across a remarkably broad range of biotin concentrations. In minimal medium lacking biotin, the strain showed little or no detectable growth, confirming its

auxotrophic phenotype. Supplementation with increasing concentrations of biotin progressively restored growth over more than five orders of magnitude of biotin concentration. To assess practical applicability, commercial soybeans with a known biotin concentration (Watanabe et al., 2014) and a biotin supplement (Jizokugata Biotin, DHC Corporation, Tokyo, Japan; product no. 32847) were analyzed using paper disks. For the soybean sample, the biotin concentration estimated from the halo size was 0.024 mg/L, whereas the expected concentration of the sample was 0.087 mg/L. For the biotin supplement, the estimated concentration was 0.65 mg/L, compared with the expected concentration of 0.50 mg/L (Figure 1). These results confirmed that the assay provides sufficient accuracy for practical use. They demonstrate that *B. subtilis* var. natto can convert environmental biotin concentrations into quantifiable growth responses without any genetic manipulation of biotin uptake or biosynthetic pathways, highlighting its utility as a simple agar-based biotin bioassay system for the analysis of complex biological samples.

Detectable growth responses were observed even at extremely low biotin concentrations (0.001 mg/L), while robust growth was maintained under substantially higher concentrations (200 mg/L). This broad responsiveness resembled the behavior previously reported for engineered *E. coli* and *C. glutamicum* biotin-sensing strains

126 lacking endogenous biotin biosynthesis and transport systems (Ikeda et al., 2013;
127 Finkenwirth et al., 2014). In contrast to those engineered systems, however, the natto
128 strain used in this study required no genetic manipulation.

129 The naturally broad biotin responsiveness of natto strains may reflect adaptation to
130 nutrient-limited fermentation environments. Natto fermentation occurs under conditions
131 of dense microbial growth and intense nutrient competition, where efficient scavenging
132 of trace vitamins could provide a selective advantage. The naturally evolved
133 auxotrophic lifestyle of natto strains may therefore have promoted highly efficient
134 biotin acquisition systems or physiological responses that enable survival across
135 fluctuating environmental biotin concentrations.

136 Further studies will be necessary to clarify the molecular mechanisms underlying the
137 broad biotin responsiveness observed in natto strains. In particular, characterization of
138 biotin transport systems, vitamin-scavenging pathways, and responses to biotin
139 precursors may provide insights into the ecological adaptation of natto strains and
140 improve the utility of microbial vitamin biosensors. Although this topic was beyond the
141 scope of the present study, which aimed to establish a biosensor system without genetic
142 modification, biotin transporters have not yet been clearly identified in natto strains
143 (Finkenwirth et al., 2013; Warneke et al., 2024). The biotin sensor described here may

144 provide a useful platform for identifying and characterizing such transport systems in
145 the future.
146
147 **Acknowledgments**
148 The authors thank Koh Nakagawa, Asako Sato and Mei Kobayashi for their technical
149 assistance. This work was supported by the industry-academia collaborative research
150 funds through the Tsuruoka Gastronomic Innovation Project subsidized by the Cabinet
151 Office, Government of Japan through the Regional University and Industry
152 Revitalization Grant Program, JST SPRING Grant Number JPMJSP2122, JSPS
153 KAKENHI Grant Number 24K21257 (Grant-in-Aid for Challenging Research
154 (Pioneering)), and research funds from Yamagata Prefecture and Tsuruoka City.
155
156 **References**
157 Anagnostopoulos, C. and Spizizen, J. (1961) Requirements for transformation in
158 *Bacillus subtilis*. *J. Bacteriol.*, **81**, 741–746.
159 Finkenwirth, F., Kirsch, F., and Eitinger, T. (2013) Solitary BioY proteins mediate
160 biotin transport into recombinant *Escherichia coli*. *J. Bacteriol.*, **195**, 4105–4111.
161 Finkenwirth, F., Kirsch, F., and Eitinger, T. (2014) A versatile *Escherichia coli* strain
162 for identification of biotin transporters and for biotin quantification. *Bioengineered*,
163 **5**, 129–132.
164 Ikeda, M., Miyamoto, A., Mutoh, S., Kitano, Y., Tajima, M. et al. (2013) Development
165 of biotin-prototrophic and -hyperauxotrophic *Corynebacterium glutamicum* strains.
166 *Appl. Environ. Microbiol.*, **79**, 4586–4594.

Kamada, M., Hase, S., Fujii, K., Miyake, M., Sato, K. et al. (2015) Whole-genome sequencing and comparative genome analysis of *Bacillus subtilis* strains isolated from non-salted fermented soybean foods. *PLoS ONE*, **10**, e0141369.

Kubo, Y., Rooney, A. P., Tsukakoshi, Y., Nakagawa, R., Hasegawa, H. et al. (2011) Phylogenetic analysis of *Bacillus subtilis* strains applicable to natto (fermented soybean) production. *Appl. Environ. Microbiol.*, **77**, 6463–6469.

Lin, S. and Cronan, J. E. (2011) Closing in on complete pathways of biotin biosynthesis. *Mol. Biosyst.*, **7**, 1811–1821.

Livaniou, E., Costopoulou, D., Vassiliadou, I., Leondiadis, L., Nyalala, J. O. et al. (2000) Analytical techniques for determining biotin. *J. Chromatogr. A*, **881**, 331–343.

Sasaki, M., Kawamura, F., and Kurusu, Y. (2004) Genetic analysis of an incomplete bio operon in a biotin auxotrophic strain of *Bacillus subtilis* natto OK2. *Biosci. Biotechnol. Biochem.*, **68**, 739–742.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M. et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods*, **9**, 676–682.

Tong, L. (2013) Structure and function of biotin-dependent carboxylases. *Cell. Mol. Life Sci.*, **70**, 863–891.

Wang, C., Chen, J., Tian, W., Han, Y., Xu, X. et al. (2023) Natto: a medicinal and edible food with health function. *Chin. Herb. Med.*, **15**, 349–359.

Warneke, R., Herzberg, C., Klein, M., Elfmann, C., Dittmann, J. et al. (2024) Coenzyme A biosynthesis in *Bacillus subtilis*: discovery of a novel precursor metabolite for salvage and its uptake system. *mBio*, **15**, e0177224.

Watanabe, T., Kioka, M., Fukushima, A., Morimoto, M. and Sawamura, H. (2014) Biotin content table of select foods and biotin intake in Japanese. *Int. J. Anal. Bio-Sci.*, **2**, 109–125.

Figure

Fig. 1. Broad-range biotin-dependent growth response of Bacillus subtilis var. natto.

A. Representative agar plate bioassay images showing growth zones formed in response to increasing biotin concentrations (0–200 mg/L). The numbers in the lower right corner of each image indicate the halo size (mm). Particularly at biotin concentrations below 0.001 mg/L, blotting paper improved the resolution of halo size differences. The third row shows the measurement results obtained using soybeans and a biotin supplement with known biotin concentrations. The estimated concentrations shown in the third row were calculated from halo sizes using the calibration curve shown in (B). B. Quantification of halo size in the agar diffusion assays. Values for paper disk represent the mean ± SD of three independent biological replicates (n = 3), whereas blotting paper was evaluated in a single experiment. Statistical significance for paper disk data was determined using Welch's t-test. Comparisons were performed between 0.0001 and 0.01 mg/L, 0.001 and 0.01 mg/L, and 100 and 200 mg/L biotin concentrations; *$P < 0.05$, **$P < 0.01$, and §$P = 0.058$. The R^2 values for blotting paper and paper disk were obtained from linear regression analyses over the ranges of 0.001–100 mg/L and 0.01–100 mg/L, respectively. The symbols (◆) and (◇) indicate the measured biotin concentrations of the soybean sample and the biotin supplement, respectively. Blotting paper enabled discrimination of biotin-dependent growth responses over five orders of magnitude.

A

Biotin concentration (mg/L)

	0	0.0001	0.001	0.01	0.1	1	10	100	200
30°C, 42h Blotting paper	7.92	8.01	28.12	35.97	39.72	51.22	58.30	70.99	79.35
30°C, 42h Paper disk	9.58	9.64	10.12	10.78	31.52	47.14	62.59	78.15	81.33

◆ Soybean 19.40 ◇ Supplement 43.04

	Soybean	Supplement
Expected biotin conc. (mg/L)	0.087	0.500
Estimated biotin conc. (mg/L)	0.024	0.650

B