Note

Growth Inhibitory Effect of Shelf Life Extending Agents on *Bacillus subtilis* IAM 1026

**SAORI MITSUBOSHI^{1*}, RIE OBITSU¹, KANAKO MURAMATSU²,
KENTARO FURUBE³, SHIGEHIRO YOSHITAKE³, AND KAN KIUCHI¹**

¹*Faculty of Home Economics, Kyoritsu women's University,
2-2-1 Hitotsubashi, Chiyoda-ku, Tokyo 103-8437, Japan*

²*Department of Human Life and Environmental Science, Niigata Women's College,
471 Ebigase, Higashi-ku, Niigatashi, Niigata 950-8680, Japan*

³*Eisai Food & Chemical Co. Ltd., 5th Floor Nihonbashi-Sunrise-Building,
2-13-10 Nihonbashi, Chuo-ku, Tokyo 103-0027, Japan*

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Natural shelf life extending agents and sugar fatty acid esters that might inhibit the growth of *B. subtilis* IAM 1026 were screened, and the effective agents were as follows: β -thujaplicin (Hinokitiol) and chitosan, inhibited the growth of IAM 1026 at a concentration of 0.001%; ϵ -polylysine and M-1695 (a sugar fatty acid ester) at 0.005%; citrus seed extract, thiamin lauryl sulfate, and grapefruit seed extract at 0.01%; CT-1695 and L-1695 (sugar fatty acid esters) at 0.05%; pectin digests and SM-800 (a sugar fatty acid ester) at 0.5%; water pepper seed extract and the sugar fatty acid esters SM-1000 and CE-1695 at 1.0%. The growth inhibitory effects of the agents in custard cream were not necessarily similar to those in liquid culture. The agent that showed the highest inhibitory effect in custard cream was 0.3% β -thujaplicin, followed by 0.3% ϵ -polylysine.

Key words : *Bacillus subtilis* IAM 1026 / Growth inhibitory effect of natural shelf life extending agents and sugar fatty acid esters / Growth inhibition in liquid culture and custard cream.

Current trends toward decreasing the salt and sugar content of foods, as well as the development of new types of food products, have made food preservation increasingly challenging (Katsumata et al., 2004; Takahashi et al., 2005; Kanai and Aida, 2006). The use of preservation agents other than salt and sugar has become increasingly important, but consumer resistance to the use of synthetic food additives has limited food manufacturers' choice of preservatives. Thus, new, more sophisticated techniques are needed for the management of food hygiene and preservation.

In this study, we screened various natural food additives and emulsifiers for bacteriostatic effects against *B. subtilis* IAM 1026 (abbreviated as IAM

1026 hereafter) and compared their growth inhibitory effects against IAM 1026 inoculated into two systems: a liquid culture medium and custard cream. The pH of the nutrient broth (Difco, NB medium) was adjusted to 7.0 after food additives were added. A loopful of bacterial cells was inoculated into 10 ml of nutrient broth and cultured by shaking reciprocally at 30°C for 24 h. The cultured medium (0.1%) was newly inoculated into 10 ml of NB medium and cultured by shaking reciprocally at 30°C.

The shelf life extending agents (Japan Food Additives Association, 1999) used were grapefruit seed extract (Daiichi Chemicals, abbreviated as GF), chitosan (Taiyo Chemical Industry, Co., Ltd.; Deacetylation degree, 85%), pectin digests (Asama Chemicals, trade name Noipectin-L, consisting of pectin digests at 28%, lactic acid at 14%, other food materials at 58%), citrus seed extract (Morita Foods,

*Corresponding author. Tel : +81-3-3237-2483, Fax : +81-3-3237-2688.

abbreviated as citrus), β -thujaplicin (trade name Hinokitiol, Seiwa Chemicals), water pepper seed extract (Sanyo Fine Chemicals, abbreviated as water pepper), thiamin lauryl sulfate (trade name Vitagen AS5, Tanabe Seiyaku, abbreviated as thiamin), and ϵ -polylysine (Chisso, 50% content, abbreviated as polylysine). The emulsifiers used were sugar fatty acid esters (Mitsubishi Chemical Foods, trade name Ryo-to Sugar Ester), L-1695, CE-1695, M-1695, CT-1695, SM-800, and SM-1000.

Growth in NB medium was assessed by absorbance at 660 nm by Mini Photo (Taitec Co.) and calculated as follows: The growth ratio is the percentage ratio of growth in the presence of a shelf life extending agent or emulsifier to the growth in the absence of that agent. Thus, the growth ratio (%) of IAM 1026 =

$$\frac{\text{Growth when a shelf life extending agent or emulsifier is added (OD}_{660})}{\text{Growth when a shelf life extending agent or emulsifier is not added (OD}_{660})} \times 100$$

The criteria for judging the growth ratio (%) and growth inhibitory effect (–to +++) were as follows: Growth ratio 0% = growth inhibitory effect +++; 0% ~ < 10% (4.70×10^7 cfu/ml), +++; 10% ~ < 40% (1.88×10^8 cfu/ml), ++; 40% ~ < 60% (2.82×10^8 cfu/ml), +; 60% ~ 90% (4.23×10^8 cfu/ml), $\pm$; 90% ~ < 100% (4.70×10^8 cfu/ml), –. Therefore, the smaller the growth ratio, the greater the inhibitory effect.

Preparation was performed according to the methods reported previously (Osawa et al., 1998).

Some agents promoted growth of IAM 1026 when present in low concentrations but inhibited growth when present at high concentrations in liquid culture. At low concentrations, these agents or their metabolic derivatives may serve as nutrients for *B. subtilis*. Fig. 1 shows the growth of IAM 1026 when GF, water pep-

per, polylysine, chitosan, or β -thujaplicin was added. The growth ratio with 0.005% GF reached 171.2% at 24 h but decreased slightly at later time points (Fig. 1A). However, the growth ratio was 3.0% at 48 h when 0.01% GF was added, and the inhibitory effect with 0.1% was +++. Addition of thiamin at the 0.005% level produced an 83.3% growth ratio, with a 103.7% growth ratio at 72 h (data not shown). With 0.01% thiamin, no growth was observed, which meant that the growth inhibitory effect was +++. Some agents showed a growth inhibitory effect at high concentrations but did not promote growth at low concentrations (Fig. 1B). Citrus is an example. The addition of 0.001% citrus inhibited growth at 24 h, but regrowth was observed at 48 h, and the growth ratio reached to 98.1% at 72 h (data not shown). 0.1% and 0.5% of water pepper inhibited growth for 48 h, but the growth ratios reached 45.0% and 48.0%, respectively, at 72 h. At a 1.0% concentration, water pepper completely inhibited growth. The growth inhibitory effect of water pepper seemed weaker than the effects of pectin digests, GF, or thiamin. Growth was inhibited almost completely by 0.005% polylysine, 0.001% chitosan, and 0.001% β -thujaplicin; thus, their growth inhibitory effects were +++ (Fig. 1C). These three shelf life extending agents were not degraded to nutrients and thus could exert strong bacteriostatic effects at low concentrations.

Addition of 0.1% SM-800 led to a high growth ratio of 143.0% at 48 h, and 0.05% SM-1000 produced a 231.0% growth ratio at 24 h (Fig. 2A). At these concentrations, both of these substances failed to inhibit the growth of IAM 1026 at later time points. Concentrations of 0.5% or higher of SM-800 inhibited growth completely. A concentration of 0.1% of SM-

FIG. 1. The growth of *Bacillus subtilis* IAM1026 with the addition of GF, water pepper, polylysine, chitosan, or β -thujaplicin.

(A), GF, $\blacktriangle$, 0.005%; $\triangle$, 0.01% : (B), water pepper; $\circ$, 0.1%; $\square$, 0.5%; $\blacklozenge$, 1.0% : (C), polylysine, $\blacktriangle$, 0.005%; chitosan, $\triangle$, 0.001%; β -thujaplicin, $\blacklozenge$, 0.01%.

1000 did not inhibit growth at 72 h, but growth was completely inhibited by a 1.0% concentration of the same substance. Since those two sugar esters contained capric or caprylic acid at 99% concentration, they might act as nutrients at low concentrations.

As shown in Fig. 2B, the growth ratio was less than 10% at a 0.05% or higher concentration of CT-1695 and the growth ratio was 62 to 69% between 24 and 72 h after 0.5% addition of CE-1695 (data not shown).

Fig. 3 shows sugar fatty acid esters that had a high growth inhibitory effect. A 0.005% addition of M-1695 produced a growth ratio of less than 3.0%, and the growth inhibitory effect was +++ (Fig. 3A). A 0.05% or higher concentration of L-1695 produced a growth ratio of less than 3.0%, with a growth inhibitory effect of +++ (Fig. 3B).

In some instances, shelf life extending agents with

growth inhibitory effects in liquid media do not have as great an effect when added to foods at the same concentrations (Katsumata et al., 2003a, b; Katsumata et al. 2004). The growth inhibitory effects of shelf life extending agents in liquid culture were investigated by adding them to custard cream at 0.1 to 1.0%. The sugar fatty acid esters were completely ineffective in custard cream, with no growth inhibitory effect even at a 1.0% concentration.

The growth inhibitory effects of chitosan, β -thujaplicin, water pepper, and polylysine in custard cream were investigated. When β -thujaplicin was added at 0.1%, the growth ratio of IAM 1026 was 11.1% (growth inhibitory effect ++) at 48 h and 21.7% (++) at 72 h, which was insufficient to prevent putrefaction. As shown in Table 1, 0.3% β -thujaplicin inhibited growth completely.

Polylysine, at a concentration of 0.3%, decreased

FIG. 2. The growth of *Bacillus subtilis* IAM1026 with the addition of SM-800, SM-1000 and CT-1695. (A), SM-800, ○, 0.1%; □, 0.5%; SM-1000, ▲, 0.05%; ●, 0.1%; ◆, 1.0%. (B), CT-1695, ●, 0.05%; ○, 0.1%; ◆, 1.0%

FIG. 3. The growth of *Bacillus subtilis* IAM1026 with the addition of M-1695 and L-1695. (A), M-1695, ▲, 0.005%; △, 0.01%. (B), L-1695, ●, 0.05%; ○, 0.1%

TABLE 1. Growth inhibitory effect of shelf life extending agents in custard cream.

Shelf life extending agents	Concentration							
	0.1%		0.3%		0.5%		1.0%	
	48 h	72 h	48 h	72 h	48 h	72 h	48 h	72 h
Chitosan	++	±			+++	—	++++	++++
β -Thujaplicin	++	++	++++	++++	++++	++++	++++	++++
Water pepper	±	+			+	—	++++	++++
Polylysine	±	—	++	+++	+++	++++	++++	++++

See the accompanying report for an explanation of the symbols.

the growth ratio to 26.3% at 48 hours (++) and 1.1% at 72 h (+++). A 0.5% addition of polylysine decreased it to 1.3% (++) at 48 h and 0.0% at 72 h (++++).

The growth ratio was higher than 100.0% when 0.5% of chitosan or water pepper was added, indicating that neither of these agents was suitable for use as a growth inhibitor at this concentration. Our results indicate that the greatest growth inhibitory effects were shown with β -thujaplicin and chitosan in liquid culture and with β -thujaplicin and polylysine in custard cream.

Putrefaction with *B. subtilis* can cause an unpleasant odor as had occurred in the putrefied custard cream in this study. We searched for natural food additives and emulsifiers that could act as shelf life extending agents to inhibit the growth of IAM 1026 at 30°C for a few days. The agents that we studied required higher concentrations to exert a growth inhibitory effect in custard cream than in a liquid medium. Kanai (1991) has reported similar results. Some agents that were effective in liquid culture were ineffective in custard cream. Thus, the effectiveness of a growth inhibitor in liquid culture may be only a rough indicator of its effectiveness in foods. The most effective shelf life extending agents in liquid culture in our study were β -thujaplicin and chitosan, which showed complete growth inhibition of IAM 1026 at a concentration of 0.001%. Since β -thujaplicin emits a unique strong flavor, it should be used without coming into direct contact with the target foods, for example in the covering film, and it should be removed by vaporization before using the foods. Shelf life extending agents other than β -thujaplicin did not emit strong flavors. However, sugar fatty acid esters are acrid, and care should therefore be taken when using them. Some shelf life extending agents, such as pectin digests, GF, and thiamin, and sugar fatty acid esters, such as SM-800 and SM-1000, promote growth of IAM 1026 at low concentrations. Addition of such levels of these substances to foods should be avoided if a growth inhibitory effect is desired. In the many foods that can be putrefied by *B. subtilis* like IAM

1026, shelf life could be extended by using some of the agents investigated here.

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