J. Microbiol. Biotechnol. (2005), **15**(6), 1178–1182

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Prediction on the Stability of Spray-Dried *Lactobacillus reuteri* KUB-AC5 by Arrhenius Equation for Long-Term Storage

KORAKOCH, HAMSUPO[1]*, PRAKIT SUKYAI[1], GÉRARD LOISEAU[2], SUNEE NITISINPRASERT[1], DIDIER MONTET[2], AND PENKHAE WANCHAITANAWONG[1]

[1]*Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand*
[2]*Centre de coopération Internationale en Recherche Agronomique pour le Développement, Département AMIS, UR Tropiqual, TA 40/16, 73 rue Jean-François Breton, 34398 Montpellier Cedex 5, France*

Received: July 10, 2004
Accepted: March 31, 2005

Abstract Survival of thermotolerant *Lactobacillus reuteri* KUB-AC5 in 20% (w/v) skim milk was found to be 11.3% after spray drying by using a pilot scale spray dryer with inlet temperature at 170°C and outlet temperature at 85°C. The ability of dried cell to produce antimicrobial activity was not affected by the spray drying. The model system for predicting viability of spray-dried *L. reuteri* KUB-AC5 during long-term storage was established, based on the Arrhenius equation, and verified by experimental data, because the viability of cells during storage can be correlated with storage temperature. The viability during storage at 30°C declined more rapidly than that storage at 4°C.

Key words: Probiotic, antimicrobial activity, spray drying, *Lactobacillus reuteri*, storage

Antibiotics have effectively been used for decades to prevent the infection of pathogenic bacteria (*Escherichia coli* and *Salmonella* sp.) responsible for chicken sickness [22]. However, the increasing dosage of antibiotics causes the development of resistant bacterial strains in poultry, which makes subsequent use of antibiotics and therapy difficult. Both consumer and manufacturer have greatly been concerned about the side-effects of antibiotics on farm animals, and therefore, are looking for alternatives [7]. One way to solve these problems is to keep the animal in good health by balancing intestinal microorganisms with the help of probiotics [24]. Lactic acid bacteria have been used in animals such as chicken and pig, because of their ability to produce varieties of antimicrobial substances. Their implementation should reduce risks of growth and survival of pathogens and spoilage microorganisms [19, 10, 11, 21].

*Corresponding author
Phone: 66-43-362-121; Fax: 66-43-362-121;
E-mail: korham@kku.ac.th

The approach to improve food and feed safety through the use of probiotics involves the development of a dried form of lactic acid bacteria. Spray drying is potentially a useful process for large-scale production of some human and animal probiotics that are based on *Lactobacillus* strains. Spray drying has been estimated to be low-cost [5]. Dried and stable starter cultures with high population of viable and uninjured cells are also needed. Previous studies reported the use of spray drying to preserve dairy starter cultures such as *Lactobacillus* sp. and *Streptococcus* sp. [5, 23, 13, 25, 8, 3, 9]. However, little information is available on the death kinetics during preservation of spray dried cells. The aim of this study was to describe a procedure to develop a model system, based on the Arrhenius equation, to predict the stability of spray-dried antimicrobial substances-producing *Lactobacillus reuteri* KUB-AC5 [18] for long-term storage.

Materials and Methods

Microorganisms

Lactobacillus reuteri KUB-AC5 isolated from some chicken intestine samples collected around Bangkok was obtained from the collection of the Department of Biotechnology, Faculty of Agro-industry, Kasetsart University, Thailand [18]. This strain was preserved in MRS medium (Difco, Detroit, MI, U.S.A.) containing 20% (v/v) glycerol at −80°C. Culture was propagated twice in MRS medium (initial pH 6.5, 18–24 h, 37°C) prior to use as the inoculum.

Spray Drying

Overnight culture of 5% (v/v) *L. reuteri* KUB-AC5 in MRS broth was inoculated into the production medium [sucrose 2%; yeast extract 0.5%; K_2HPO_4 0.2%; $(NH_4)_2SO_4$ 0.1%;

tween 80 0.1%; $MgSO_4 \cdot 7H_2O$ 0.01%; $MnSO_4 \cdot 4H_2O$ 0.005%] and incubated at 37°C until the stationary growth phase was reached. Then, the culture was mixed with 20% (w/v) non-sterilized skim milk powder (Lovelait, France). The cell suspension was directly spray dried in the pilot scale spray dryer (Anhydro No. 3, Denmark) with a nominal water evaporation capacity of 27 kg/h, 1.6 m diameter, and 4 m height. A pump delivered the feed solution to a stainless steel atomizer with a rotary atomizer. The moisture in spray droplets was evaporated in a vertical, counter-current drying chamber. For spray drying conditions, the air temperatures at the inlet and outlet of the spray dryer were adjusted to 170°C and 85°C, respectively. The powder was collected in a single cyclone separator. Each sample of spray-dried *L. reuteri* KUB-AC5 was analyzed for survival rate, moisture content, and antimicrobial activity. Dried samples were also stored in sealed plastic bags at 4, 30, 37, and 45°C to investigate their stability.

Viable Cell Number

The number of viable cells was counted on an MRS agar plate. Each sample of spray-dried *L. reuteri* KUB-AC5 (0.1 g) was rehydrated with 10 ml of 0.85% NaCl solution by vigorous shaking for 2 min. The rehydrated sample was serially diluted in 0.85% NaCl solution and plate-counted on 1.5% MRS agar in duplicate. MRS plates were then incubated at 37°C for 24 h. The rate in percent of surviving bacteria of each sample after spray drying was calculated as follows: % survival rate=$(N/N_0)\times 100$, where N_0 is initial viable cells before spray drying (CFU/g of solids), N is the number of viable cells after spray drying (CFU/g of solids), and CFU/g of solids is the colony forming unit of viable cells per gram of spray-dried powder.

Moisture Content

The moisture content of spray-dried powders was determined in a drying oven at 105°C according to Official Methods of Analysis [1].

Antimicrobial Activity Determination

Rehydrated samples of spray-dried *L. reuteri* KUB-AC5 were propagated twice using MRS broth at 37°C. Then, cell-free supernatants of MRS broth cultured overnight were examined for antimicrobial activity by the spot-on-lawn method [15]. Briefly, serial twofold dilutions of cell-free supernatant were spotted (10 mml) onto fresh indicator plates. These indicator plates were prepared by overlaying 5 ml of soft NA agar (0.75% agar) with 10 ml of the indicator strain at the concentration of about 10^7 CFU/ml. *Salmonella* sp. from infectious chicken, obtained from Betagro Agro-Group Public Company Limited, Samutprakarn, Thailand [18], was used as an indicator strain. Then, the overlaid agar plates were incubated at 30°C for 6 h. The inhibition area was revealed by the formation of clear zones in the indicator bacterial lawn. Antimicrobial activity was expressed in arbitrary units (AU) per ml of the original cultures, calculated as follows: AU/ml is the highest dilution exhibiting inhibition zone per volume of spotting supernatant.

RESULTS AND DISCUSSION

Pilot Scale Spray Drying of *L. reuteri* KUB-AC5

The preliminary spray drying experiment was performed at air inlet of 170°C and outlet temperatures of 85°C, to evaluate the survival rate of *L. reuteri* KUB-AC5 in 20% (w/v) skim milk. The survival of *L. reuteri* KUB-AC5 was 11.3% (from 1.5×10^9 to 1.7×10^8 CFU/g of solids). Viability of all replicates decreased after spray drying. It was most likely due to dehydration and thermal damage of cell structures and cell components during spray drying [25, 12]. In addition, thermal shock may occur during the introduction of spray droplets into the hotter inlet air, which could also have an effect on cell survival [13]. Our results are similar to those of many researchers who reported about the survival rate of *Lactobacillus* sp. in skim milk powder after spray drying: Espina and Packard [6] obtained a survival rate of 8.2% of *L. acidophilus* (inlet and outlet temperatures of 170°C and 75°C), Mauriello *et al.* [16] indicated that the survival of spray-dried *L. curvatus* 32Y was 10% (inlet and outlet temperatures of 160°C and 68°C), and Gardiner *et al.* [9] also reported that the survival rate of *L. paracasei* NFBC 338 was 12.8% after spray drying at air inlet and outlet temperatures of 180°C and 72°C, respectively. However, it is difficult to compare the survival rates after spray drying of different studies, because each study employed different microorganisms or model systems such as membranes, liposomes, or enzymes [2].

Antimicrobial activity of spray-dried *L. reuteri* KUB-AC5 was also determined. The spray-dried cells were repropagated in MRS broth, and the MRS broth culture was assayed for production of antimicrobial substances. The results showed that the cells were able to resuscitate and exhibited 200 AU/ml of antimicrobial activity. There were no differences between the ability of antimicrobial substance production of *L. reuteri* KUB-AC5 before and after spray drying. Therefore, spray drying had no effect on the ability of this strain to produce antimicrobial substances. Similar results were also obtained by Mauriello *et al.* [16], Morgan *et al.* [17] and Gardiner *et al.* [8], who reported that the spray drying process did not affect the ability of lactic acid bacteria to produce bacteriocin.

Survival of Spray-Dried *L. reuteri* KUB-AC5 During Storage

The viability of spray-dried *L. reuteri* KUB-AC5 was evaluated during storage at 4°C or 30°C for 118 days (Fig. 1). It is obvious that the storage temperature was a critical parameter

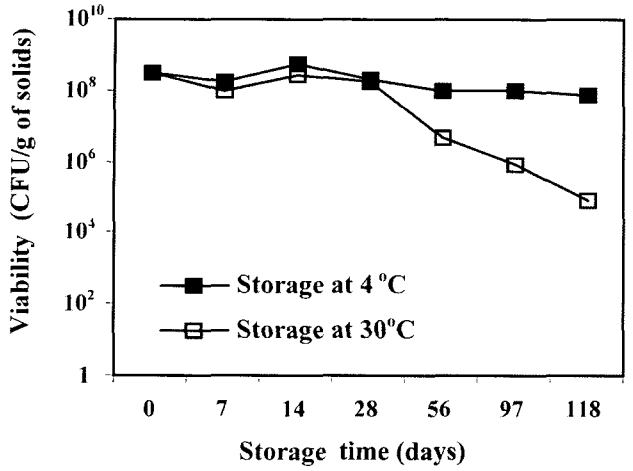

Fig. 1. Viability of spray-dried *L. reuteri* KUB-AC5 during storage at 4°C and 30°C.

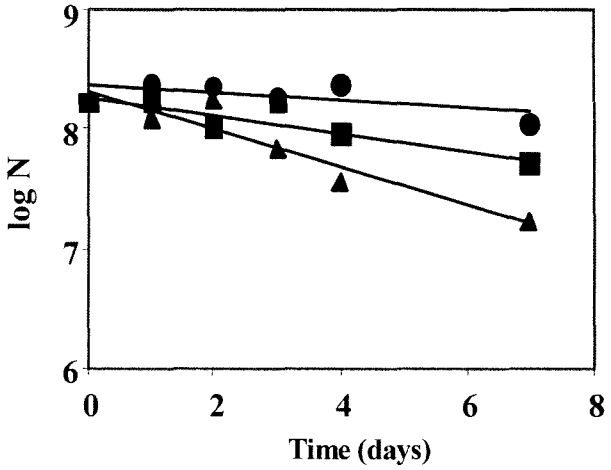

Fig. 2. Viability of spray-dried *L. reuteri* KUB-AC5 stored at 30°C, 37°C, and 45°C as a function of time. Circles: 30°C, logN=8.3652-0.0312t, Squares: 37°C, logN=8.2719-0.0742t, Triangles: 45°C, logN=8.3011-0.1557t.

affecting the survival of the spray-dried cells. The viability of the samples was quite stable during storage at 4°C for 4 months, and the total number of viable bacteria decreased as the storage temperature increased to 30°C. Olsen *et al.* [20] reported that the survival of dried lactic acid bacteria was strongly dependent on the storage temperature, and that storage stability increased with decreasing temperature. Similarly, Espina and Packard [6] showed that the percentage of survival decreased immediately (about 50% loss) after storage of spray-dried *L. acidophilus* at 4°C for 30 days. Gardiner *et al.* [9] also reported that probiotic viability at 4°C and 15°C was maintained at about 10^8 CFU/g for up to 49 days, while viability at 30°C declined to 10^6 CFU/g during 49 days of storage. During storage at 4°C and 30°C, the spray-dried *L. reuteri* KUB-AC5 retained its ability to produce antimicrobial substances at the same level (200 AU/ml) throughout the storage for 118 days.

Prediction on the Stability of Spray-Dried *L. reuteri* KUB-AC5 by Accelerated Storage Test

An accelerated storage test was used to predict the long-term preservation of lactic acid bacteria [4]. The stability of spray-dried *L. reuteri* KUB-AC5 was examined by incubation of the spray-dried *L. reuteri* KUB-AC5 preserved in 20% (w/v) skim milk, packed in sealed plastic bags, and kept at 30°C, 37°C, and 45°C for 7 days. The specific rate of degradation (k) of microorganisms can be determined from Equation 1 [4, 14].

$$\log N=\log N_0-kt \quad (1)$$

where N_0 is the initial viable cells (CFU/g of solids), N is the viable cells at any time (CFU/g of solids), k is the specific rate of degradation (h^{-1}), and t is the storage time.

Therefore, k of spray-dried *L. reuteri* KUB-AC5 for three different temperatures can be calculated for each temperature from the slope of the straight lines in Fig. 2 and the factor k is shown in Table 1.

Thermal degradation (Eq. 2) of microorganisms follows the logarithmic form of the Arrhenius equation with respect to absolute temperature [14].

$$\log k=-\left[\frac{\Delta Ha}{2303R}\right]\left[\frac{1}{T}\right] \quad (2)$$

where k is the specific rate of degradation (h^{-1}), Ha is the heat of activation ($J mole^{-1}$), R is the gas constant (8.32 $J mole^{-1}\cdot K$), and T is the absolute temperature (K).

Consequently, the Arrhenius graph was plotted from the specific rate of degradation (Table 2) in terms of logarithm (log k) versus the reciprocal of the absolute temperature (1/T), as shown in Fig. 3.

Therefore, the specific rate of degradation at 4°C was estimated from the Arrhenius equation in Fig. 3 ($k_4=1.286\times 10^{-3}\ h^{-1}$). The prediction of the viability of spray-dried *L. reuteri* KUB-AC5 for long-term conservation at 4°C and 30°C was given by replacement of the value of N_0 and the factors k_4 and k_{30} in Equation 1, as shown in Equation 3 and Equation 4, respectively.

$$\log N=\log N_0-1.286\times 10^{-3}t \quad (3)$$

$$\log N=\log N_0-0.031t \quad (4)$$

Table 1. Specific rate of degradation at 30°C, 37°C, and 45°C of spray-dried *L. reuteri* KUB-AC5.

Storage temperature (°C)	Specific rate of degradation, k (h^{-1})
30	0.031
37	0.074
45	0.156

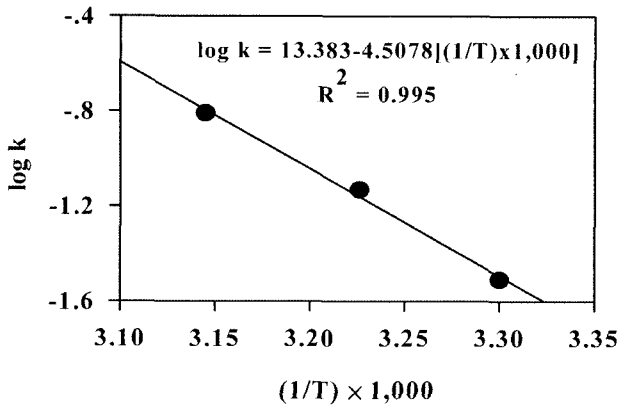

Fig. 3. Arrhenius plot of the specific rate of degradation (k) of spray-dried *L. reuteri* KUB-AC5 (logk versus 1/T×1,000).

Comparison of prediction and experimental survival rates of spray-dried *L. reuteri* KUB-AC5 at 4°C and 30°C did not show any significant differences for 4 months (Fig. 4). The results showed that the accelerated storage test was an acceptable extrapolation tool of the stability at 4°C and 30°C. The activation energy calculated from the Arrhenius equation is a good indicator to compare thermoresistance and death rate of microorganisms during spray drying and freeze drying, even though it is not a true value [13, 14].

In conclusion, the thermotolerant *L. reuteri* KUB-AC5 in dry form would be useful for the development of new probiotic ingredients in the field of feed stuffs. The objective to stabilize the lactic acid bacteria *L. reuteri* KUB-AC5 by spray drying while preserving its antimicrobial activity was achieved. The survival of the bacteria after spray drying was estimated to be 11.3% of the bacteria initially present. The spray-dried *L. reuteri* KUB-AC5 retained its ability to produce antimicrobial substances after storage at

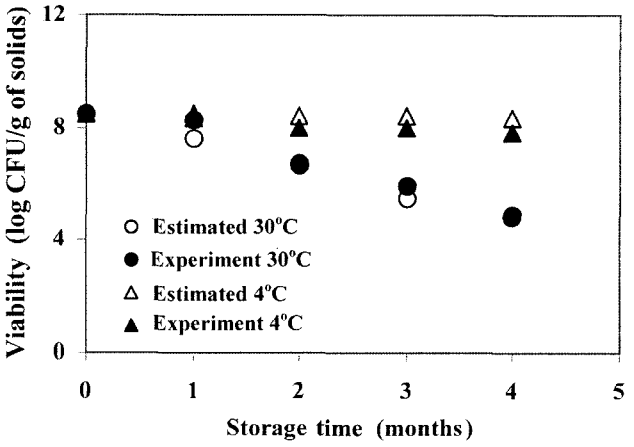

Fig. 4. Comparison of the estimated and the experimentally measured viabilities of spray-dried *L. reuteri* KUB-AC5 during storage at 4°C and 30°C.

4°C and 30°C for 4 months. Temperature is a critical factor for microbial survival during spray drying and storage. The accelerated storage test is advantageous because of its rapidity. Therefore, using the Arrhenius equation to develop a model system to predict the stability of spray-dried cells appears to be an interesting prediction and comparison tool to study the viability during storage at 4°C and 30°C.

Acknowledgments

The study was supported by the Commision on Higher Education, Ministry of Education, Royal Thai Government and French-Thai cooperative project. The authors thank the staff of Cirad-Amis (Montpellier, France) as well as IUT (Montpellier, France) for providing the spray dryer used in this study.

References

1. AOAC. 2000. *Official Methods of Analysis*. 17th Ed. AOAC International, Gaithersburg, Maryland, U.S.A.
2. Carvalho, A. S., J. Silva, P. Ho, P. Teixeira, F. X. Malcata, and P. Gibbs. 2002. Survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage in the presence of protective agents. *Biotech. Lett.* **24:** 1587–1591.
3. Desmond, C., C. Stanton, G. F. Fitzgerald, K. Collins, and R. P. Ross. 2001. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. *Int. Dairy J.* **11:** 801–808.
4. Desmons, S., H. Krhouz, P. Evrard, and P. Thonart. 1998. Improvement of lactic cell production. *Appl. Biochem Biotech.* **70/72:** 513–526.
5. Desmons, S., S. Zgoulli, P. Evrard., D. Roblain, J. Destain, and P. Thonart. 1998. Spray-drying of different lactic acid species. *Med. Fac. Landbouww. Univ. Gent.* **63/4a:** 1253–1261.
6. Espina, F. and V. S. Packard. 1979. Survival of *Lactobacillus acidophilus* in a spray-drying process. *J. Food Prot.* **42(2):** 149–152.
7. Fuller, R. 1989. Probiotics in man and animals. *J. Appl. Bacteriol.* **66:** 365–378.
8. Gardiner, G. E., E. O'Sullivan, J. Kelly, M. A. E. Auty, G. F. Fitzgerald, J. K. Collins, R. P. Ross, and C. Stanton. 2000. Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. *Appl. Environ. Microbiol.* **66(6):** 2605–2612.
9. Gardiner, G. E., P. Bouchier, E. O'Sullivan, J. Kelly, J. K. Collins, G. Fitzgerald, R. P. Ross, and C. Stanton. 2002 A spray-dried culture for probiotic cheddar cheese manufacture. *Int. Dairy J.* **12(9):** 749–756.
10. Garriga, M., M. Pascual, J. M. Monfort, and M. Hugas. 1998. Selection of lactobacilli for chicken probiotic adjuncts. *J. Appl. Microbiol.* **84:** 125–132.

11. Gusils, C., A. P. Chaia, S. González, and G. Oliver. 1999. Lactobacilli isolated from chicken intestines: Potential use as probiotics. *J. Food Prot.* **62(3):** 252–256.
12. Johnson, J. A. C. and M. R. Etzel. 1995. Properties of *Lactobacillus helveticus* CNRZ-32 attenuted by spray-drying, freeze-drying, or freezing. *J. Dairy Sci.* **78:** 761–768.
13. Kim, S. S. and S. R. Bhowmik. 1990. Survival of lactic acid bacteria during spray drying of plain yogurt. *J. Food Sci.* **55(4):** 1008–1010.
14. Lee, J. M. 1991. Sterilization, pp. 213–239. *In Biochemical Engineering.* Prentice Hall, Englewood Cliffs, New Jersey, U.S.A.
15. Leroy, F. and L. De Vuyst. 2002. Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. *Int. J. Food Microbiol.* **72:** 155–164.
16. Mauriello, G., M. Aponte, R. Andolfi, G. Moschetti, and F. Villani. 1999. Spray-drying of bacteriocin-producing lactic acid bacteria. *J. Food Protect.* **62(7):** 773–777.
17. Morgan, S. M., M. Galvin, J. Kelly, R. P. Ross, and C. Hill. 1999. Development of a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. *J. Food Prot.* **62(9):** 1011–1016.
18. Nitisinprasert, S., V. Nilphai, P. Bunyun, P. Sukyai, K. Doi, and K. Sonomoto. 2000. Screening and identification of effective thermotolerant lactic acid bacteria producing antimicrobial activity against *Escherichia coli* and *Salmonella* sp. resistant to antibiotics. *Kasetsart J. (Nat. Sci.).* **34:** 387–400.
19. Nousianien, S. and J. Setälä. 1998. Lactic acid bacteria as animal probiotics, pp. 437–473. *In* S. Salminen. and A. V. Wright (eds.), *Lactic Acid Bacteria: Microbiology and Functional Aspects*, 2nd Ed. Marcel Dekker, New York, U.S.A.
20. Olsen, E. S., T. Sørhaug, S. E. Birkeland, and R. Pehrson. 1999. Survival of *Lactobacillus helveticus* entrapped in Ca-alginate in relation to water content, storage and rehydration. *J. Ind. Microbiol. Biotech.* **23:** 79–85.
21. Ouwehand, A. C. 1998. Antimicrobial components from lactic acid bacteria, pp. 139–159. *In* Salminen, S. and A. V. Wright (eds.), *Lactic Acid Bacteria: Microbiology and Functional Aspects*, 2nd Ed. Marcel Dekker, New York, U.S.A.
22. Perry, F. G. 1995. Biotechnology in animal feeds and animal feeding: An overview, pp. 1–15. *In* Wallace, R. J. and A. Chesson (eds.), *Biotechnology in Animal Feeds and Animal Feeding.* Weinheim, New York, U.S.A.
23. Porubcan, R. S. and R. L. Seller. 1975. Spray drying of yogurt and related cultures. *J. Dairy Sci.* **58(5):** 787.
24. Tannock, G. W. 1999. Introduction, pp. 1–14. *In* Tannock, G. W. (ed.), *Probiotics: A Critical Review.* Horizon Scientific Press, Wymondham, U.K.
25. Teixeira, P. C., M. H. Castro, F. X. Malcata, and R. Kirby. 1995. Survival of *Lactobacillus delbrueckii* ssp. *Bulgaricus* following spray-drying. *J. Dairy Sci.* **78:** 1025–1031.