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Different Growth Media and Growth Phases Affecting on Spray Drying and Freeze Drying of *Lactobacillus reuteri* KUB-AC5

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ABSTRACT

To develop a probiotics adjunct, spray drying and freeze drying of *Lactobacillus reuteri* KUB-AC5 grown in different growth media and growth phases were determined. After cultivation, the viable cell of culture in Basal medium was approximately 1 log cycle lower than that in MRS broth. The highest survival rate after spray drying (92.5%) was obtained from the culture grown in Basal medium for 12 h while the cultivation of culture in MRS broth for 12 h gave the highest survival rate after freeze drying (86.7%). *Lb. reuteri* KUB-AC5 at stationary phase was more resistant to stress of drying than exponential phase cultures. It was found that the ability of dried cell to produce antimicrobial substances was not affected by drying processes and storage. During storage at 4°C for 2 months, the viable cell of spray dried cells decreased approximately 1 log cycle while the viable cell of the freeze dried cells were quite stable.

Key words: *Lactobacillus reuteri*, spray drying, freeze drying, antimicrobial activity

INTRODUCTION

Lactic acid bacteria (LAB) are widely used as starter cultures in the production of food, dairy products and feed (Knorr, 1998; Mäyrä-Mäkinen and Bigret, 1998). In addition, LAB are also important for human and animal health. LAB have been used as probiotics to improve the normal flora of the host intestine because of their ability to produce varieties of antimicrobial substances including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide, bacteriocin and other metabolites (Helander *et al.*, 1997). Expanding interest in ready-to-use culture concentrates for the direct inoculation has placed greater emphasis on starter production and preservation methods that promote high cell viability and activity (Broadbent and Lin, 1999). Maximization of survival of LAB cultures during drying and subsequent storage for long periods is importan, both technologically and economically. Since many steps are required to produce dried cultures, suitable selection of both the growth media and growth phases is essential to study and need a critical parameter to prepare dried LAB cultures (Teixeira *et al*., 1995a; Gardiner *et al.*, 2000; Carvalho *et al.*, 2003a).

The aim of this work was to develop a procedure of dried cell production for commercial use as probiotic adjuncts by study on the impacts of growth media and growth phases on survival of *Lactobacillus reuteri* KUB-AC5 and its antimicrobial activity during spray drying, freeze drying and subsequent storage at 4°C.

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MATERIALS AND METHODS

1. Microorganism

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 (Nitisinprasert *et al*., 2000). This strain was preserved at –80°C in MRS medium (Difco) containing 20% (v/v) glycerol. Culture was propagated twice in MRS medium (initial pH 6.5,18-24 h, 37°C) prior to use as the inoculum.

2. Cultures preparation

5% (v/v) inoculum were propagated statically in flask containing MRS broth (De Man *et al*., 1960) or the Basal medium (Modified after Somjaipeng 2001). The cultures were cultivated at 37°C for 6 h and 12 h. The compositions of the media were given in Table 1.

3. Drying processes

The cultivated cultures were resuspended in 20% (w/v) skim milk powder (Himedia) as drying medium. For spray drying, the cell suspension was directly spray dried with a laboratory spray dryer (Buchi B-191, Switzerland). The air temperatures at the inlet and outlet of the spray dryer were adjusted at 110°C and 70°C, respectively. For freeze drying, the cell suspension were frozen by rotation in a bath of 95% ethyl alcohol (-20°C). Then, the frozen samples were freeze dried for 24 h with a freeze dryer at vacuum of 1.5 mbar, -80°C (Heto FD4, Belgium). Each sample of dried *Lb. reuteri* KUB-AC5 was analyzed for survival rate, moisture content and antimicrobial activity. Dried samples were also stored in sealed polypropylene (PP) plastic bags at 4°C to investigate their stability. The experiments were repeated twice.

Table 1 Composition of the media used for growth of *Lb. reuteri* KUB-AC5.

Component	MRS broth	Basal medium
Glucose	20 g	-
Sucrose	-	20 g
Beef extract	10 g	-
Peptone	10 g	-
Yeast extract	5 g	5 g
Tween 80	1 ml	1 ml
K_2HPO_4	2 g	2 g
$(NH_4)_2SO_4$	-	1 g
Sodium acetate	5 g	-
Tri-ammonium citrate	2 g	-
$MgSO_4 \cdot 7H_2O$	0.2 g	0.1
$MnSO_4 \cdot 4H_2O$	0.2 g	0.05 g
Distilled water	1000 ml	1000 ml

4. Analysis

Viable cell number. The number of viable cells was counted on a MRS agar plate. Each sample of dried *Lb. 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 percentage of cells survival of each sample after drying was calculated as follows: % survival = $(N/N_0) \times 100$, where N_0 is initial viable cells before drying (CFU/g of solids) and N is number of viable cells after drying (CFU/g of solids).

Moisture content. Moisture content of dried powders was determined in drying oven at 105°C according to AOAC Official method 960.18 (AOAC, 2000).

Antimicrobial activity determination. Rehydrated samples of dried *Lb. reuteri* KUB-AC5 were propagated twice using MRS broth at 37°C. Then, cell-free supernatant of overnight MRS broth were examined for antimicrobial activity by the spot-on-lawn method (Leroy and De Vuyst, 2002). Briefly, serial twofold dilutions of cell-free supernatant were spotted (10 µl) onto

fresh indicator plates. These indicator plates were prepared by overlaying 5 ml of soft NA agar (0.75% agar) with 10 µl of the indicator strain at the concentration of about 10^7 CFU/ml. *Salmonella* sp. S003 from infectious chicken, obtained from Betagro Agro-Group Public Company Limited, Samutprakarn, Thailand (Nitisinprasert *et al*., 2000) was used as indicator strain. Then, the overlaid agar plates were incubated at 30°C, 6 h. Inhibition area was revealed by the formation of clear zone 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 ml of spotting supernatant.

Detection of the antimicrobial activity of the substances in powder product. About 10^3 CFU/ml of the indicator strain, *Salmonella* sp. S003 was grown in nutrient broth (Difco) without and with 1 and 3% (w/v) dried *Lb. reuteri* KUB-AC5. After incubation at 30°C for 6 h, survival of the indicator strain was determined by the nutrient agar plate method.

RESULTS AND DISCUSSION

Survival rate of *Lb. reuteri* KUB-AC5 after spray drying and freeze drying

In this study, *Lb. reuteri* KUB-AC5 was grown in two different media (MRS broth and Basal medium). The culture cultivations were carried out until reached to the exponential growth phase (6 h) and the stationary growth phase (12 h). These cultures were applied for spray drying and freeze drying processes. Then, the dried cell powders were determined for survival rate, moisture content and antimicrobial activity after drying

The results in Table 2 showed the effect of different growth media on viable cell of *Lb. reuteri* KUB-AC5 after cultivation and drying. The viable cell of the culture in MRS broth gave approximately 1 log cycle higher than that in Basal medium after cultivation. MRS broth supply of nutritive and growth substances more than Basal medium (Table 1). Therefore, this difference might explain the good growth of *Lb. reuteri* KUB-AC5 in MRS broth. Unfortunately, MRS broth is an

Table 2 Number of *Lb. reuteri* KUB-AC5 in various cultivated conditions before and afterspray drying and freeze drying.

Characteristics	Spray drying				Freeze drying			
	MRS 6 h	MRS 12 h	Basal 6 h	Basal 12 h	MRS 6 h	MRS 12 h	Basal 6 h	Basal 12 h
1. Viable cell after cultivation (CFU/ ml)	1.5×10^9	1.96×10^9	5.30×10^8	7.50×10^8	1.80×10^9	2.40×10^9	5.95×10^8	7.4×10^8
2. Viable cell before drying (CFU/g of solids)	6.35×10^9	8.35×10^9	2.78×10^9	3.20×10^9	9.00×10^9	1.20×10^{10}	2.98×10^9	3.73×10^9
3. Viable cell after drying (CFU/g of solids)	5.30×10^9	7.05×10^9	2.32×10^9	2.96×10^9	5.05×10^9	1.04×10^{10}	2.90×10^8	1.82×10^9
4. Survival rate (%)	83.46	84.43	83.45	92.50	56.11	86.67	9.73	48.79
5. Moisture content (%)	2.63	4.46	1.88	2.72	2.58	2.83	0.64	0.39
6. The ability of the strain to produce antimicrobial substances (AU/ml) before/after drying	200/200	200/200	200/200	200/200	200/200	200/200	200/200	200/200

expensive medium as compared with the Basal medium so the cost of MRS broth would likely restrict its large scale industrial use. Desmons *et al*. (1998a) suggested that a culture medium providing an optimal growth with maximum economy would be of great interest for industrial use. However, the survival rate of *Lb. reuteri* KUB-AC5 were slightly decreased after spray drying. It was certainly due to dehydration and thermal damaged of cell structures and cell components during spray drying (Teixeira *et al*., 1995b). In addition, thermal shock may occur during the introduction of spray droplets into the hotter inlet air, which could have also an effect on cell survival (Kim and Bhowmik, 1990). For freeze drying, the viable cell number of all samples decreased may result from freezing and dehydration. The major factors that cause the injury might be cold shock, change of cell wall permeability and metabolic injury (Johnson and Etzel, 1995).

From Table 2, it was found that the survival rate after drying of stationary phase cultures (12 h) was higher than the exponential phase cultures (6 h). It is probably because cells at stationary phase are more resistant to stress than exponential phase cultures (Johnson and Etzel, 1995). The results showed that growth media and growth phases had effected the survival rate of *Lb. reuteri* KUB-AC5 after freeze drying. The mechanisms underlying damage and protection by freeze drying are indeed complex and not fully understood to date (Carvalho *et al.*, 2003b).

The antimicrobial activity of *Lb. reuteri* KUB-AC5 before and after drying processes was also evaluated. The results showed that the dried cells were able to resuscitate and exhibited 200 AU/ml of antimicrobial activity (Table 2). Therefore, spray drying and freeze drying had no effect on the ability of this strain to produce antimicrobial substances. Similar results were obtained by Mauriello *et al.* (1999) and Gardiner *et al.* (2000), who reported that spray drying process did not affect the ability of lactic acid bacteria to produce bacteriocin.

Inhibition of *Salmonella* sp. S003 growth by antimicrobial substances in powder product was presented in Figure 1. The dried powders were still active against *Salmonella* sp. S003. As the amount of dried powder increased from 1 to 3%, the viable cell number of *Salmonella* sp. S003 was reduced ranging from 1 to 3 log cycles. This result revealed that the antimicrobial substances in the spray dried

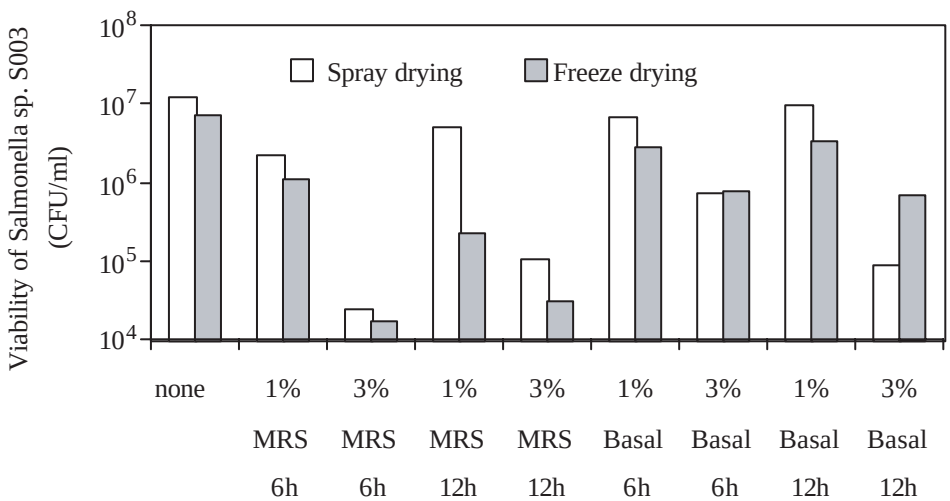

Figure 1 Viable cell number of *Salmonella* sp. S003 after incubating in nutrient broth at 30°C for 6 h without (none) and with 1 and 3% (w/v) spray dried and freeze dried *Lb. reuteri* KUB-AC5.

and freeze dried *Lb. reuteri* KUB-AC5 products still exhibited the antimicrobial activity.

Stability of spray dried and freeze dried *Lb. reuteri* KUB-AC5

Stability of the dried *Lb. reuteri* KUB-AC5 in various growth conditions during storage at 4°C for 2 months were shown in Figure 2. It was observed that the survival rate of the freeze dried cells was quite stable. Particularly, there was no decline in the survival rate during storage of the freeze dried cultures which were grown in Basal medium. Carvalho *et al.* (2004) suggested that sugar in the growth medium also plays a critical role in survival during storage of freeze dried cells, which interacts with the drying medium. Moreover, Zayed and Roos (2004) reported that the freeze dried cells in skim milk mixed with sucrose gave a higher survival rate during storage than that in skim milk or sucrose separately. Therefore, it was possible that residual sucrose concentration in Basal medium approximately 3-5 g/L after the cultivation of *Lb. reuteri* KUB-AC5 for 6-12 h (Table 3) might be responsible for survival rate during storage of freeze dried *Lb. reuteri* KUB-AC5. For the spray dried cells, the survival rate had been a gradual decrease during storage. However, a difference of the viable cell of spray dried cells after storage for 2 months were approximately decreased only 1 log cycle (from 10^9 to 10^8). The survival rate of spray dried cells (15-18%) during storage were lower than the survival rate of freeze dried cells (67-100%) may be due to the high moisture content (Figure 3), which could be negative for long-term preservation (Desmons *et al*., 1998b). The low in inlet (110°C) and outlet (70°C) temperatures of the spray drying process would result in wet powder. Therefore, the moisture content of spray dried cells was higher than the moisture content of freeze dried cells. Actually, the mechanism of cell death during storage in the dried state are still unknown but decreasing of fatty acid content of cell membrane and the degree of lipid oxidation

Table 3 The residual sucrose concentration in Basal medium of *Lb. reuteri* KUB-AC5 growthin Basal medium without controlled pH at 37°C.

Cultivation time (h)	The residual sucrose concentration (g/l)
0	20.0
2	16.1
4	7.5
6	4.4
8	3.4
10	3.2
12	3.0

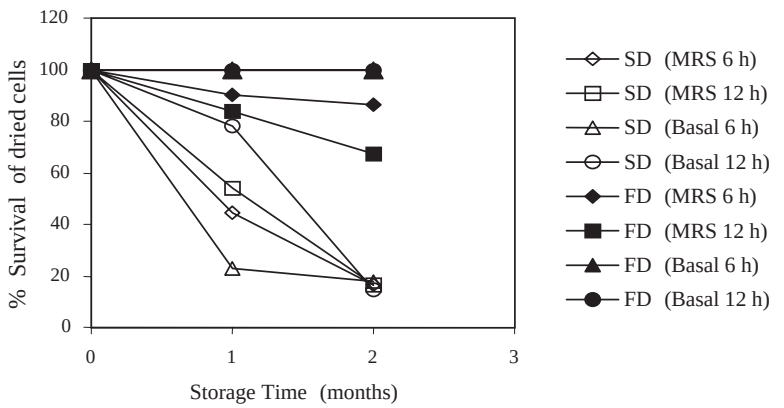

Figure 2 Survival rate of spray dried (SD) and freeze dried (FD) *Lb. reuteri* KUB-AC5 in various cultivated conditions during storage at 4°C for 2 months.

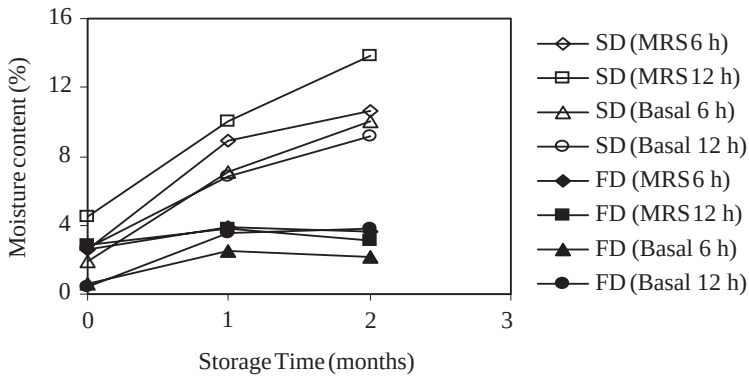

Figure 3 Moisture content of spray dried (SD) and freeze dried (FD) *Lb. reuteri* KUB-AC5 in various cultivated conditions during storage at 4°C for 2 months.

were apparently related to decrease the cell survival during storage (Teixeira *et al*., 1996). In addition, the dried *Lb. reuteri* KUB-AC5 retained its ability to produce antimicrobial substances at the same level (200 AU/ml) throughout storage at 4°C for 2 months.

CONCLUSIONS

The results of this study demonstrated that dried cells of *Lb. reuteri* KUB-AC5 could be produced by spray drying and freeze drying. *Lb. reuteri* KUB-AC5 at stationary phase (12 h) was more resistant to stress of drying than exponential phase (6 h) cultures. Cultivated cells at 12 h in Basal medium and MRS broth gave the highest survival rate after spray drying (92.5%) and freeze drying (86.7%), respectively. Under storage at 4°C for 2 months, the dried powders preserved its initial activity and the viable cell of freeze dried cells especially the culture in Basal medium were quite more stable than the spray dried cells. For the purpose of drying in large quantities of bacterial cultures, Basal medium would be preferable to MRS broth due to cheap cost of its composition.

The thermotolerant *Lb. reuteri* KUB-AC5 in dry form would be useful for the development of new probiotic ingredient in the field of food and feed stuffs. Therefore, it would be further study in industrial scale to assure that the desirable survival rate of dried cells were achieved. In addition, study on optimizing drying and stability of *Lb. reuteri* KUB-AC5 such as the protective agents, others storage temperatures and packaging should also be concerned.

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