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Cultivation of *Lactobacillus* sp. and Production of Probiotic Powder as Animal Feed Additive

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Abstract: The objectives of this study were to develop culture medium and fillers with appropriate growth conditions and to evaluate stability of selected *Lactobacillus salivarius* L61 and commercial probiotic (*L. plantarum* Fr-B). Both strains were cultured in full-fat Soybean, Skim Milk, Dextrose (SSMD) and K_2HPO_4 with 1, 5 or 10 g/L yeast extract compared to de Man, Rogosa and Sharpe (MRS) medium. Stabilities of powdered *Lactobacillus* cultures were examined using Ground Rice Husk (GRH) or Rice Bran (RB) fillers with 25% (w/w) $CaCO_3$. *Lactobacillus* counts were determined using ten-fold serial dilution of a pour plate method on MRS agar and incubated in anaerobic jar at 37°C for 48 h. The results showed that *Lactobacilli* populations in MRS broth were higher than those in SSMD but had lower stability. *Lactobacillus salivarius* L61 and *L. plantarum* Fr-B population growth at optimal incubation time (24 h) and optimal growth medium were 2.63 x 10^{10} CFU/ml and 1 x 10^9 CFU/ml. The optimal growth media (w/v) was 1% full-fat soybean, 1% skim milk, 0.5% yeast extract, 0.5% dextrose and 0.05% K_2HPO_4. The stability of *Lactobacillus* L61 in GRH was higher than in RB and the stability under refrigeration temperature (RFT, 4-6°C) in both fillers were higher those in room temperature (RMT, 28-30°C). The GRH filler could maintain 10^9 CFU/g of *Lactobacillus* L61 cells at RFT and at RMT for 35 and 21 d, respectively while RB could maintain 10^9 CFU/g of *Lactobacillus* L61 cells at RFT for 14 d.

Key words: *Lactobacillus*, production, probiotic powder

INTRODUCTION

Probiotic microbes are used for health benefits in humans and animals. When applying probiotic microbes for animal production, it is necessary to prepare the biomass and check the stabilities of microbes during storage. The optimal number of Lactic Acid Bacteria (LAB), in a dried culture probiotic form, is indicated to be not less than 10^6 CFU/g before administering to animals (Kailasapathy and Chin, 2000; Anadon *et al*., 2006). The viability of probiotic microbes depends on many factors, e.g., strain, medium composition and storage technology. Biomass production of *Lactobacillus* probiotic groups uses a specific medium, commonly consisting of an energy source (carbon source), growth factors and buffering capacity (Sornplang and Uriyapongson, 2007). The main carbon source is short-chain sugar such as maltose, sucrose, glucose or fructose. Growth factors are indicated by nitrogen sources, the majority of which consist of peptides and amino acids. In addition, growth factors include minerals, vitamins and dietary fiber. Storage technologies for probiotics are classified into two main forms, liquid culture and dried culture. The liquid culture form of probiotics is easy to prepare and can be utilized immediately but the shelf life is reduced. On the other hand, dried culture forms, such as freeze-dried and spray-dried, require many preparation steps but the shelf life is increased. Powder culture production is one form of dried culture with decreased preparation steps and which is used as an alternative for culture storage of Lactic Acid Bacteria (LAB) probiotics. Therefore, the objectives of this study were to develop a culture medium for biomass production of the selected *Lactobacillus* strain and to investigate the probiotic shelf life when stored under refrigeration and at room temperature using powder culture preparation for animal production.

MATERIALS AND METHODS

Bacterial strains and bacteria identification: *Lactobacillus* L61 was isolated from native chicken feces and qualified as a probiotic according to our previous study (Sornplang and Leelavatcharamas, 2010). It was identified at a species level using 16S rRNA gene sequences. The region of the 16S rRNA gene of lactobacilli DNA was amplified using the primers SU forward (5'-CAC CAA CAG AGT TTG ATC CTG GCT CAG-3') and HDA2 reverse (5'-GTA TTA CCG CGG CTG CTG GCA-3'), as described by Tannock *et al.* (2000). *Lactobacillus plantarum* Fr-B strain, isolated

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from a commercial probiotic product, was obtained from the Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University Bangkok, Thailand.

Media and culture conditions: The selected strain and commercial probiotic were incubated under microaerophilic conditions at 37°C for 48 h. Full plates of bacterial colonies were harvested and inoculated into 1 L of de Man, Rogosa and Sharpe broth (MRS; Oxoid, England), or a 1.2 L bottle containing 1 L of autoclaved and cooled full-fat soybean-skim milk-dextrose (SSMD)-based medium (full-fat soybean 10 g, skim milk 10 g, dextrose 5 g, $K_2H(PO)_4$ 0.5 g) with different yeast extract concentrations. Bacterial growth in the basal medium was supplemented by adding various concentrations, 0.1, 0.5 and 1% (w/v), of yeast extract. Samples were then incubated at 37°C for 48 h. Bacterial growth in a basal medium containing 0.5% (w/v) yeast extract with or without the addition of 0.5% (w/v) dextrose was also studied. Fermentations were agitated every 6 h using a magnetic stirrer at 50 rpm. Biomass yields of cultures were investigated using a spectrophotometer at 600 nm (OD_{600}). A ten-fold serial dilution method using a sterilized 0.1% peptone solution was utilized to determine the number of Colony-Forming Units (CFU) in 1 ml of basal medium. *Lactobacillus* counts were determined using a pour plate method on MRS agar (Oxoid, England) after incubation in an aerobic chamber at 37°C for 48 h.

Stability of powdered culture: Twenty ml of fresh *Lactobacillus* cultures with maximal cells (10^{10} CFU/ml) were mixed with 80 g of either rice husk or rice bran filler as primary mix cultures, following the method of Sriprasertsak (1981) modified by adding 20 g of $CaCO_3$. The primary mix cultures were mixed homogeneously for 10 min. Viabilities of powdered *Lactobacillus* cultures were measured weekly; samples were kept for 35 d at room temperature and at refrigerator temperature (4-6°C). Powdered samples were diluted in Maximum Recovery Diluent (MRD) and plated in MRS agar to enumerate cells. Stabilities of powdered cultures were established by the percentage of cells remaining viable during storage. Samples were tested in duplicate.

RESULTS AND DISCUSSION

16S rRNA gene sequences: *Lactobacillus* L61 was identified as *L. salivarius* using 16S rRNA gene sequences. It had high similarity (99-100%) compared to GenBank (accession number CP000233.1)

Effect of culture medium on bacterial growth: Following a 9 h lag phase, *Lactobacillus* L61 and *L. plantarum* Fr-B attained maximum cell growth after 24 h of incubation time in SSMD with various yeast extract concentrations. Both strains were unable to increase cell counts after 36 h of fermentation because of early cell deaths (Table 1, 2). Growth of both strains in the full-fat Soybean-Skim Milk (SSM)-based medium with the addition of dextrose was gradually improved with the amount of yeast extract added. The addition of 1% (w/v) yeast extract to the SSMD medium led to the highest cell numbers of both strains (Table 1, 2). However, the addition of 0.5% (w/v) yeast extract could develop a high cell count (10.42 log CFU/ml) in the *Lactobacillus* L61 strain after 24 h of incubation. In this study, to reduce costs of the medium and to ensure sufficient numbers of *Lactobacillus* cells (≥9 log CFU/ml) for mixing into the feed of broiler chickens, only 0.5% (w/v) yeast extract could be added to the basal medium formulation. Lactobacilli grew well in the medium containing 10% (w/v) skim milk supplemented with yeast extract concentration of 1% (w/v), as reported by Avonts *et al.* (2004). In this study, both strains of lactobacilli grew well in the medium containing soybean, a low concentration of skim milk at 0.1% (w/v) and the addition of 0.5-1% (w/v) yeast extract. The addition of 0.5% (w/v) dextrose in SSM medium supplemented with 0.5% (w/v) yeast extract resulted in high cell counts of *Lactobacillus* L61. But SSM-yeast extract medium without the addition of dextrose was unable to develop high *Lactobacillus* cell counts (Table 3). Therefore, optimal bacterial growth was obtained using a medium containing full-fat Soybean, Skim Milk, Yeast Extract, Dextrose (SSMYD) and a buffering agent, $K_2H(PO)_4$, at 1, 1, 0.5, 0.5 and 0.05% (w/v), respectively.

Biomass production of the *Lactobacillus* L61 strain was lower in the SSMYD medium compared with the MRS medium. However, the number of bacterial cells was

Table 1: Cell concentrations (log CFU/ml) of biomass production of selected *Lactobacillus* L61 strain in full-fat soybean-skim milk-dextrose (SSMD) medium with various yeast extract concentrations

Incubation time (h)	Yeast extract concentrations (% w/v) 0.1	0.5	1.0
0	5.45±0.24	5.45±0.24	5.45±0.24
18	8.43±0.26	9.23±0.23	9.42±0.26
24	9.16±0.07	10.42±0.09	10.44±0.45
36	8.11±0.07	9.00±0.03	9.14±0.04
48	7.71±0.42	8.45±0.04	8.71±0.47

Table 2: Cell concentrations (log CFU/ml) of biomass production of *Lactobacillus plantarum* Fr-B strain in full-fat soybean-skim milk-dextrose (SSMD) medium with various yeast extract concentrations

Incubation time (h)	Yeast extract concentrations (% w/v) 0.1	0.5	1.0
0	5.38±0.52	5.38±0.52	5.38±0.52
18	7.93±0.46	8.75±0.47	8.93±0.54
24	8.10±0.06	9.00±0.04	9.07±0.07
36	8.04±0.17	8.98±0.15	9.04±0.06
48	7.55±0.47	8.35±0.04	8.54±0.42

Table 3: Cell concentrations (log CFU/ml) of biomass production of *Lactobacillus* L61 strain in 1% (w/v) full-fat soybean, 1% (w/v) skim milk and 0.5% (w/v) yeast extract (SSMY) medium with or without 0.5% (w/v) dextrose supplementation

Incubation time (h)	With dextrose	Without dextrose
0	5.38±0.52	5.45±0.24
18	9.42±0.26	7.10±0.05
24	10.10±0.09	6.99±0.12
36	9.14±0.04	6.94±0.46
48	8.71±0.47	6.55±0.47

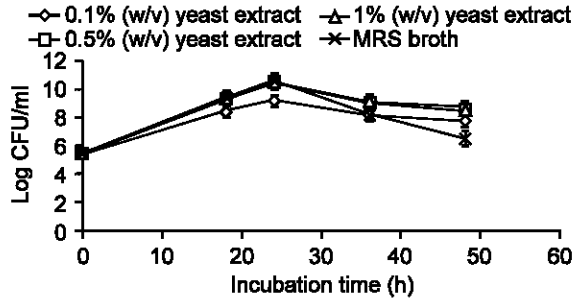

Fig. 1: Biomass production of *Lactobacillus* L61 in SSMYD medium varied yeast extract concentration and MRS broth

much more stable in the SSMYD medium (Fig. 1). This result indicates that a high concentration of glucose (20 g/L) in the MRS medium causes excessive lactic acid production which in turn leads to a decrease of viable cells (Chauvatcharin, 1999). Full-fat soybeans have high levels of high-quality protein (36-42%) and high levels of fat (18-22%) and hence are suitable for inclusion in the diets of livestock. Our previous study reported that *Lactobacillus* bacteria are fastidious regarding nutrients for growth, preferring foods such as garlic, salt and ground roasted gelatinous rice-ingredients which are also used in *pla-chom*, a fermented food product (Sornplang *et al*., 2011). This study used full-fat soybeans together with skim milk and yeast extract as an alternative carbon source to produce a high number of cells in *Lactobacillus* culture (up to 10.44 log CFU/ml). At the same time, full-fat soybeans, skim milk and yeast extract were nitrogen sources which were necessary for *Lactobacillus* growth.

Effect of adding fillers on stabilities of the powdered culture: The viabilities of powdered *Lactobacillus* L61 prepared by the addition of ground rice husk or rice bran as a filler are shown in Table 4. The powdered *Lactobacillus* L61 added to both these fillers and stored under refrigeration had higher cell survival rates than the powders stored at room temperature (Fig. 2). Ground rice husk and rice bran as filler in *Lactobacillus* L61 culture maintained cells at 10^9 CFU/g at refrigerator temperature for 35 and 14 d of storage, respectively

Table 4: Survival of cells (log CFU/g)[1] of *Lactobacillus* L61 in the primary mix culture with ground rice husk or rice bran added

	Storage		
Filler type	Time (d)	Refrigerated (4-6°C)	Room temperature
Ground rice husk	0	10.42±0.01	10.44±0.01
	7	10.37±0.01	10.33±0.01
	14	10.33±0.01	10.19±0.01
	21	9.93±0.01	9.90±0.01
	28	9.92±0.01	8.71±0.01
	35	9.89±0.01	7.41±0.01
Rice bran	0	10.40±0.01	10.37±0.01
	7	10.35±0.01	8.10±0.01
	14	9.67±0.04	6.72±0.01
	21	8.34±0.02	5.49±0.02
	28	7.51±0.01	4.52±0.01
	35	6.55±0.01	2.71±0.01

[1]Values are means±SD in duplicate

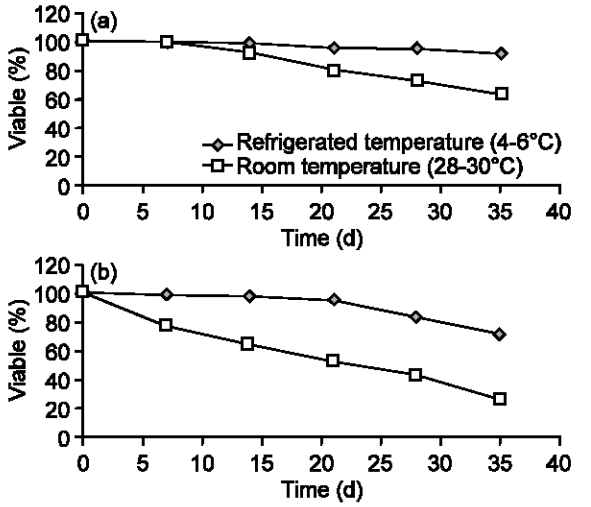

Fig. 2: The viability of Lactobacillus L61 strain in fillers stored refrigerated temperature and at room temperature for 35 d. A = Rice husk, B = Rice bran

(Table 4). Powdered *Lactobacillus* L61 with added ground rice husk maintained cells at 10^9 CFU/g at room temperature for 21 d. Using rice bran as a filler caused a decrease in cells to 10^8 CFU/g at 7 d and a continuous decrease to 10^2 CFU/g at 35 d (Table 4). This indicates that the high temperature was absorbed due to the high fat content (12-13%) of rice bran, causing the destruction of *Lactobacillus* cells-similar to results reported by Nitisinprasert *et al*. (2005).

Conclusions: In biomass production of *Lactobacillus* probiotics, MRS medium can be replaced by SSMYD medium containing 1% (w/v) full-fat soybean, 1% (w/v) skim milk, 0.5% (w/v) yeast extract and 0.5% (w/v) dextrose, supplemented with 0.05% (w/v) $K_2H(PO)_4$, a buffering agent. In the SSMYD medium, higher cell

numbers of the *Lactobacillus* L61 strain could be developed compared with a commercial probiotic. The shelf life of the powder culture of the *Lactobacillus* probiotic was improved by adding filler (rice husk or rice bran). The stability of *Lactobacillus* L61 in both fillers stored under refrigeration was greater than those stored at room temperature. Rice husk and rice bran as filler maintained the *Lactobacillus* L61 at 10^9 CFU/g at refrigerator temperature for 35 and 14 d of storage, respectively.

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REFERENCES

Anadon, A., M.R. Martinez-Larranaga and M.A. Martinez, 2006. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul. Toxicol. Pharmacol., 45: 91-95.

Avonts, L., E. VanUytven and L.De Vuyst, 2004. Cell growth and bacteriocin production of probiotic *Lactobacillus* strains in different media. Int Dairy J., 14: 947-955.

Chauvatcharin, S., 1999. Development of the cultivation of high cell density of lactic acid bacteria: application of metabolic control. Department of Biotechnology, Faculty of Science, Mahidol University. Reported research. TRF.

Kailasapathy, K. and J. Chin, 2000. Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. Immunol Cell Biol., 78: 80-88.

Nitisinprasert, S., P. Wanchaitanawong, N. Amornthewaphat and S. Chuenchom, 2005. Effect of probiotic "*Lactobacillus reuteri* KUB-AC5" to the growth of chicken. Reported research. National Research Council of Thailand.

Sornplang, P. and S. Uriyapongson, 2007. Viability of lactic acid bacteria and utilization as probiotic in animals. Khon Kaen Agr J., 35: 277-286.

Sornplang, P. and V. Leelavatcharamas, 2010. Antimicrobial susceptibility of probiotic lactobacilli isolated from chicken feces. Khon Kaen Univ. Res. J., 15: 689-697.

Sornplang, P., V. Leelavatcharamas, P. Sukon and S. Yowarach, 2011. Antibiotic resistance of lactic acid bacteria isolated from a fermented fish product, *pla-chom*. Res. J. Microbiol., 6: 898-903.

Sriprasertsak, P., 1981. Production and preservation of powder lactic acid bacteria for swine feed supplement.Master of Science Thesis in Microbiology. Graduate School, Kasetsart University, Thailand.

Tannock, G.W., K. Munro, H.J.M. Harmsen, G.W. Welling, J. Smart and P.K. Gopal, 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Applied Environ. Microbiol., 66: 2578-2588.