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Maximization of Growth and Storage of Locally Isolated *Lactobacillus Salivarius Subsp. Salivarius* with High Stability and Functionality

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Abstract— **This study, conforms the protocol adopted to agree with bio-safety aspects of *Lactobacillus salivarius subsp. salivarus* including evaluation of viability during storage and incubation of free cells under starving conditions for optimization of various process parameters such as heat, high concentration of salt, inoculums' size, and pH have been studied. A time course experiment was conducted incorporating all the optimized parameters. This study improves the production, functionality and the enlargement of the commercial availability as one of the important probiotics in drugs, food and feed. Estimated optimum conditions established for the growth of *Lactobacillus salivarius subsp. salivarus* are as follows: pH =6.5; temperature = 37-40°C inoculums size =5 % (w/v) and salt concentration = 6.0% (w/v). The storage stability of the cultures was excellent in freeze-dried form and for cultures frozen at −45°C or less for 10 months.**

Keywords— ***L. salivarius subsp. salivarius*, probiotics, improving storage, survival, validity.**

I. Introduction

Probiotics, defined as ''Live microorganisms which when administered in adequate amounts confer a health benefit on the host'' Lactobacilli are one of the most common used probiotics, it is Gram positive rods belonging to the group of Lactic Acid Bacteria (LAB).Lactobacilli, as well as some species of the genus Bifidobacteria and Streptococci, they are micro-organisms which classified in the GRAS (Generally Regarded as Safe) group. They have also been proposed as probiotics for both the gastrointestinal and urogenital tracts [1, 2]. They are beneficial bacteria, which, when administered as a part of the daily dietary intake, it reduces the incidence and severity of acute and chronic infection, facilitate prevention and reduced recurrence of certain cancers and lower the incidence of several atopic conditions [3]. They are found in the mucosl membranes of humans and animals (oral cavity, intestine and vagina), on plants and materials of plant origin. [4]. Researchers suggest that regular consumption of live *lactobacillus* bacteria can improve the gut micro flora and reduce the number of infections by reducing unwanted bacteria which beneficially affect the host by improving intestinal microbial balance and have immunomodulatory effects, by modulating cytokine levels or by antagonizing and excluding pro-inflammatory micro-organisms from the gastrointestinal tract [5]. Fermentation often improves the biological value of foods, so increasing the nutritional value for the consumer; a concentration of approximately 10^6 viable cells g^{-1} product at the time of consumption is considered functional [6].

Lactobacilli are known to require complex media containing numerous amino acids, vitamins and related growth factors in addition to fermentable carbohydrates [7, 8] Substrate exhaustion was probably associated with cell maintenance and survival [9].

This study will address specific problems associated with optimizing conditions of the most favorable growth conditions required to obtain good quality and highest biomass in the shortest possible time and good storage of probiotic *L. salivarius subsp. salivarus* by batch culture for the industrial management. This research is aimed at: 1) Optimizing production conditions for probiotic *L.salivarius subsp. salivarus*. (Temperature, pH, salt concentration and inoculums' size); 2) Characterizing the storage conditions for long periods during freezing, freeze-drying and preservation in10% (v/v) glycerol at -80 °C.

II. Materials and Methods

A. Probiotic L. salivarius subsp. salivairus

The strain of *L. salivarius subsp. salivarius* was isolated from free-range local chicken; the isolates were kept at the School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM, Malaysia.

B. Preparation of probiotic starter culture inocula

The microorganism *L. salivarius subsp. salivarius* was used throughout this study. The strain was stored at −80 °C in 10% (v/v) glycerol (BDH Laboratory Supplies, Poole, England). For inoculums preparation, the stock culture was sub-cultured in a 1-L Erlenmeyer flask containing 200 mL de Mann, Rogosa, Sharpe (MRS) broth (Merck, Darmstadt,

A. McGoron, C. Li, and W.-C. Lin (Eds.): 25th Southern Biomedical Engineering Conference 2009, IFMBE Proceedings 24, pp. 175–178, 2009.
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Germany) and incubated at 37 °C for 18hrs The culture was washed with saline solution (0.85% NaCl) to remove the spent media and resuspended in the same solution to obtain an initial biomass concentration of approximately 100 CFU mL−1. For the cell concentrate preparation, the cultures were grown in 2000 mL MRS broth and the cell mass was harvested by centrifugation at 14000×g for 10 min (SIGMA-LAB [SL]) orbital shaker. The bacterial viable counts were estimated by plating on MRS-agar (Merck) the number of colony-forming units (cfu) cfu/ml was quantified using plate count method for counting the viable *Lactobacillus* cells, after incubating anaerobically for 18 hrs at 35, 37, 40 and 45°C at pH 4.5, 5.5, 6.5, or 8 with 1N HCL or NaOH, The pH was determined with a pH meter (DEITA 320).The inoculums' size and the sodium acetate salt concentration were optimized and then the storage stability of the organism e.g. The freeze-drying, the storage of probiotic in frozen cultures at −18, , -45, -68 and -80 °C and The survival in10% (v/v) glycerol at -80 °C were evaluated and optimized. All experiments were carried out in triplicate and the mean values were reported with standard deviation.

C. Survival of freeze-dried L. salivarius subsp. salivarius

Considering that the working volume was 2000 ml and the fresh biomass for every fermentation batch ranged from 18 to 20 g, the viable population in fresh biomass was found be about 10^9 -10^{10}CFU/g. The freeze-drying probiotic powders were stored in sealed polythene bags, which were placed in aluminum coated bottles, and stored at 4 and 25°C, their viability was determined on the day of powder manufacture and during powder storage for 10 months every 30 days.

III. Results and Discussion

A. Growth rate increased with temperature

The survival of the probiotic strain at 30, 37, 40 and 45°C are shown in Table 1. All investigations attained high cell populations when incubated at 37°C and at 40°C, reaching maximum populations of 5.0-4.8 log cfu ml^{-1} in 18h. Significantly lower cell counts were obtained during fermentation at 30 °C was 3.5-3.3 log cfu ml^{-1}. However, at 45 °C it did not sustain these viable counts it declined to 2.2 log cfu ml^{-1}.Table 1.

B. Growth rate affected by the pH

For all the conditions evaluated, results show that the *L .salivareus subsp. salivarius* yields the highest growth at pH of 6.5 in MRS broth and are therefore the optimum conditions (higher final O.D. and growth rate, as well as shorter lag phase and exponential phase time). While almost a minor growth at pH 4.5–5.5 was detected. A pH below 4.4 would possibly not be favorable to the survival of the strain, Table 2.

Table 1 The biomass of *L. salivarius subsp. salivarius* in 200 mL (MRS) broth at different degrees of temperatures at (30°C, 37 °C, 40 °C and 45 °C)

Time	biomass at 30 °C	biomass at 37 °C	biomass at 40 °C	biomass at 45 °C
1st exp. 18 hrs.	3.1	4.3	4.2	2.3
2nd exp. 18 hrs.	3.5	5.0	4.4	2.5
3rd exp. 18 hrs	3.3	4.8	4.5	2.2
Mean	3.2	4.7	4.2	2.3

Table 2 The Effect of different pH concentration on the total viable counts of *L. salivareus subsp. salivarius* in MRS

Time duration	pH 4.5	pH 5.5	pH 6.5	pH 8.0
1st exp. 18 hrs	1.2	2.0	5.2	4.2
2nd exp. 18 hrs	0.8	2.8	5.0	4.0
3rd exp. 18 hrs	1.8	2.9	4.9	4.1
Mean	1.2	2.5	5.0	4.1

C. Growth rate optimized by the inoculums' size

In all inoculums' sizes evaluated, results show that the *L. salivareus subsp. salivarius* yields the highest growth at 5% (w/v) inoculums' size. Table 3.

Table 3 The biomass of *L. salivarius subsp. salivarius* at different Inoculums' sizes

Time duration	Inoculums' sizes 1% (w/v)	Inoculums' sizes 3% (w/v)	Inoculum' sizes 5% (w/v)	Inoculums' sizes 7% (w/v)
1st exp. 18 hrs	2.1	4.0	4.5	3.8
2nd exp. 18 hrs	1.9	4.2	4.9	3.5
3rd exp. 18 hrs	2.0	4.5	4.6	3.5
Mean	2.0	4.2	4.6	3.6

D. Effect of salt concentrations (sodium acetate) on the growth rate

The survival of the probiotic strain at different salt concentrations was studied and the optimum concentration found is 6.o% (w/v) according to the viable counts of the probiotic *L. salivareus subsp. salivarius* in MRS.Table 4.

As studied the optimum parameters for good growth is confirmed to be an initial pH of 6·5, inoculums' sizes 5% (w/v), salt concentration of 6.o% (w/v) and temperature of 37-40 °C as an evidenced by the higher cell mass and growth rates obtained.

Table 4 Effect of sodium acetate concentration on the total viable counts *of L. salivareus subsp. salivarius* in MRS

Time duration	Salt conc. of 4.0% (w/v)	Salt conc.of 6.o% (w/v)	Salt conc. of 8.o% (w/v)	Salt conc. of 10% (w/v)
1st exp. 18 hrs	3.3	4.9	3.5	2.2
2nd exp. 18 hrs	3.1	4.6	3.6	2.5
3rd exp. 18 hrs	3.0	5.1	3.5	2.0
Mean	3.1	4.8	3.5	2.3

E. Stability and survival of the cells during the storage process

E_1. Survival of the frozen cultures

The *L. salivareus subsp. salivarius* strain reproduced well in 200 ml fermentation broth (MRS) at 37°C, inoculums' sizes 5% (w/v) and Salt concentration of 6.o% (w/v), having cell counts of 10^2 cfu/ml. The survival was excellent during the freezing of the concentrated cultures on dry-ice, which had cell counts higher than10^3 cfu/ml. These results show that the optimization of fermentation conditions enhance the survival of *L. salivarius subsp salivarius*.

Transportation of frozen cultures generally takes place on dry-ice. The survival of frozen cultures at −18°C was poor 62.12 $\log_{10}$CFU/ml^{-1} (showing a decrease about 37.88 $\log_{10}$CFU/ml^{-1}), but improved at lower temperatures. At −45°C the cell counts were 96.52$\log_{10}$CFU mL^{-1} after 10 months. The results confirm the fact that the storage temperature needs to be at−45°C or below for the frozen cultures in order to maintain a high cell count for 10 months table 5_a.

Table 5_a The survival of the *L. salivarius subsp. salivarius* cultures during freezing (The starting biomass is 10^8 cfu/ml)

Time duration	Stored at -18 °C	Stored at -45°C	Stored at -68 °C	Stored at -80 °C
2 months	10^2M CFU/mL	10^2 M CFU/mL	10^2M CFU/mL	10^2M CFU/mL
4 months	62.12M CFU/mL	10^2 M CFU/mL	10^2M CFU/mL	10^2M CFU/mL
6 months	11.62M CFU/mL	98.22M CFU/mL	10^2M CFU/mL	10^2M CFU/mL
8 months	00.00 CFU/mL	97.00 CFU/mL	98.80 CFU/mL	99.20C FU/ mL
10months	00.00 CFU/mL	96.52 CFU/ml	98.00 CFU/mL	99.00 CFU/ mL

E_2. Survival of the freeze-dried cultures

Freezing and freeze-drying are regarded as suitable methods to preserve bacterial strains for a long period of time. The viability of L. salivarius subsp. salivarius during an extended period in MRS plus glycerol, with an average 1.52-log10 CFU ml−1 decline and 1.04-log10 CFU ml−1 decline, respectively, after 10 months of storage at -80°C. When the freeze-dried cultures were stored for 10 months at +4°C, the counts remained steady for the first two months and 95.82log10 CFU ml−1 (an average 4.18 log10) decline of viable cells per gm of culture was observed after that. And when was stored at room temperature, the results were 60.12 log10 CFU ml−1 (an average 39.88 log10) decline was observed after the 1st month. This result indicated that the strain tolerated well the period that might possibly be needed for transportation, more than 50% survival during the first two months at +25°C.

The freeze-dried starter samples of *L. salivarius subsp. salivarius* stored for 10 months at −45°C or less, and at +4°C for six months, without any significant loss in viability. The results demonstrate that freeze-drying is a very effective way of storing probiotic cultures, but the optimization of the production process is the most important factor. Optimization of fermentation conditions for a particular strain can lead to a higher survival rate of freeze-dried cultures. Storage *L. salivarius subsp. salivarius* starter cultures by the freezing

and freeze-drying by the standard method was largely successful. It was evident; however, that it would need a MRS growth medium and the optimization of the production process to improve its process stability. The storage stability of the cultures was excellent in freeze-dried form and for cultures frozen at −45°C or below for 10 months table 5_b.

Table 5_b The survival of the *L. salivarius subsp. salivarius* cultures during freeze-drying (The starting biomassis10^8cfu/ml)

Time duration	Freeze-dryingat +25°C	Freeze-drying at 4°C	Freeze-drying at -45°C	Freeze-drying at -80°C
2 months	60.12M CFU/mL	10^2 M CFU /mL	10^2M CFU/mL	10^2M CFU/mL
4 months	51.2 M CFU/ mL	95.82M CFU/mL	10^2M CFU/mL	10^2M CFU/mL
6 months	32.00M CFU/mL	92.35M CFU/mL	10^2M CFU/mL	10^2M CFU/mL
8 months	3.2 M CFU /mL	87.00M CFU/mL	99.2M CFU/mL	10^2 M CFU/mL
10months	21.26.00M CFU/mL	89.50M CFU/mL	98.73M CFU/mL	99.40M CFU/mL

E_3. The survival in10% (v/v) glycerol at -80 °C

The *L. salivarius subsp. salivarius* cultured in MRS broth during preservation in10% (v/v) glycerol at -80 °C and the probiotic count remained high (98x10^6 cfu/ml with an average 0.2-$\log_{10}$ CFU ml^{-1} decline) throughout the six-month storage period. The cells that survived this process maintained their viability quite well during storage, Table 5_c.

Table 5_c The survival of *L. salivarius subsp. salivarius* in 200 mL (MRS) broth at 37 °C for 18hrs

Time duration	10 %(v/v) glycerol at 80°C.
2 months	10^2 MCFU /mL
4 months	102 MCFU/ mL
6 months	98 MCFU /mL

*The biomass is 10^8cfu/ml = 10^2x 10^6 = 10^2 (Million)CFU/m.

VI. Conclusions

We found that the optimal growth conditions were recorded in MRS broth, with an initial pH of 6·5, salt concentration 6.0(w/v), inoculums' size 5.0 %(w/v) and a temperature of 37-40 °C in order to ensure the best survival and the storage environment of the probiotic, this was in accordance with [10]. The survival of the *Lactobacillus salivareus subsp. salivarius* as probiotic bacteria during powder storage was inversely related to storage temperature. Maximum survival was achieved for 2 months, and the excellent storage was obtained in freeze-dried and freezing form, at −45°C to−80°C for 10 months, this agreed with [11] who found good survival during storage at low temperatures.

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