Vietnam Journal of Science and Technology ***61*** *(1) (2023) 47-56*

doi:10.15625/2525-2518/16310

Factor influencing the survivability of *Tetragenococcus halophilus* CH6-2 in the spray drying process

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Received: 21 July 2021; Accepted for publication: 16 September 2022

Abstract. In this study, several factors affecting *Tetragenococcus halophilus* CH6-2 cell survival rate in spray drying process including growth conditions, protective agents, and stress adaption were investigated. *T. halophilus* CH6-2, which was grown in different media, was mixed with different protective agents or at different ratios of cell concentration to protective agents or was subjected to stress adaption before spray drying. The survival rate after spray drying and after storage was determined. The results showed that skim milk had the strongest protective effect on *T. halophilus* CH6-2 cells during spray drying. De Man, Rogosa and Sharpe (MRS) was a better medium than our developed M7 optimal medium for culturing *T. halophilus* CH6-2 in respect of survivability after storage. Increasing the cell/protective agent ratio from 1/20 to 1/3 could increase the survivability after spray drying but not during storage. The heat stress and osmotic stress in this study did not improve the cell survivability after spray drying and storage.

Keywords: cell survivability, growth conditions, heat stress, osmotic stress, protective agents.

Classification numbers: 1.1.5, 1.3.2.

1. INTRODUCTION

Fermented fish sauce is popular in East Asia, especially in Viet Nam. Many attempts have been made to shorten the fermentation time to 6 months without affecting the sensory characteristics by using proteolytic enzymes accompanied with microorganisms. *Tetragenococcus halophilus* is a halophilic lactic acid bacterium, that has been proven to have a positive effect on aroma and flavor of fish sauce when used as a starter culture [1]. *T. halophilus* CH6-2 has been reported to produce active volatile compounds and possess aminopeptidase activity [2]. To use as a starter culture for fish sauce fermentation, the powder formulation of *T. halophilus* CH6-2 is desired. Freeze and spray drying are commonly used methods. Freeze drying, also called lyophilization can preserve most of the cells, causing less damage to cells. On the other hand, spray drying is considered a good long-term preservation method that can be used on a larger scale at a relatively lower price.

Currently, little is known about drying of *T. halophilus* but there are abundant studies on drying of lactic acid bacteria that could be applied. Growth conditions, stress adaption, and protective agents are common factors that influence the survivability of cells during spray drying [3]. Maltodextrin, trehalose, sucrose, and skim milk are frequently used in the food industry due

to their natural origin, lack of toxicity and biodegradability [4]. An optimal protective agent may not always be the best for all bacteria, or suitable for both spray drying and storage [3]. The combination of different protective agents could result in enhancing the survivability of bacteria during spray drying and storage [4]. The survivability of bacteria during spray drying depended on the ratio of bacterial cells to protectants [5]. Under harsh conditions, bacteria cells use variety of strategies to survive, including the upregulation of stress response proteins, cell membrane functioning maintenance, and metabolic shifts, therefore stress adaption of bacteria before drying is another way to improve the survivability of cells [3]. Different culture media cause bacteria to be stressed in different ways. Parlindungan found that *L. plantarum* survivability was significantly reduced when being cultured in MRS without Tween 80 [6]. Osmotic stress is induced during the cultivation of cells at low water activity. Guiqiang He discovered that *T. halophilus* cultured in 12 % NaCl exhibited a higher amount of intracellular proline, betaine, and trehalose, that could provide the best protection for cells during spray drying [7], hence increasing salt concentration might be considered as a strategy to increase the survivability of bacteria. Another method to improve survivability of bacteria is heat stress. Desmond demonstrated that *L. paracasei* after heat treating at 45 to 52 ℃ for 15 minutes could better withstand spray drying [8].

This study focused on understanding the effect of growth media, ratio of cell concentration to protectants, protective agents, and stress adaption on the survivability of *T. halophilus* CH 6-2.

2. MATERIALS AND METHODS

2.1. Materials

The strain *Tetragenococcus halophilus* CH6-2 [2] isolated from Cat Hai fish sauce was kept at -20 °C in MRS broth containing 25 % glycerol. *T. halophilus* CH6-2 from glycerol cultures inoculated twice in MRS broth supplemented with 50 g/L NaCl for 3 days at 35 °C was used as inoculum.

Table 1. Protective agent systems.

Protective agents	Concentration (% w/v)
SM	20
SM+S	10+10
SM+MSG	10+10
SM+MSG+S	1+9.5+9.5
MD	20
SM+MD	10+10
S+MD	10+10
SM+MSG	19+1
	15+5
	10+10
	5+15
	1+19

Protective agents: Skim milk (SM), Maltodextrin (MD), Monosodium Glutamate (MSG), and Sucrose (S) were purchased from Bien Xanh Laboratory Equipment Co. Ltd. (Ha Noi). MD,

MSG, S, and SM were diluted in distilled water at a final concentration of 20 % with subsequent sterilization at 121 °C for 15 minutes. These solutions were mixed at the concentration of each as presented in Table 1.

2.2. Methods

2.2.1. Spray drying and storage conditions

The spray drying process of *T. halophilus* CH6-2 was carried out using a laboratory scale spray dryer (Büchi Mini Spray Dryer B-290, Switzerland). The feed solution was pneumatically atomized into a vertical, co-current drying chamber using a two-fluid nozzle at a constant flow rate of 3 mL/min. The inlet temperature was 130 °C. The outlet temperature was around 70 - 85 °C. The dried powder was collected in a single cyclone separator.

The dried powder was distributed in small plastic containers, which were then dark-stored in a cool refrigerator compartment (Panasonic Inverter 234L) at 10 °C.

2.2.2. Effect of protective agents

Inoculum from 2.1 was transferred to MRS broth medium to obtain initial optical density OD at 600 nm of 1. Cells were harvested after three days of incubation at 35 °C by centrifugation at 6000 rpm, 4 °C for 10 minutes, and were re-suspended into different protective solutions (Table 1) at a ratio of 1/20 (w/w), mixed well and spray dried.

2.2.3. Effect of cell/protective agent ratio

Prepared culture grown in MRS medium as described in 2.2 was re-suspended in SM+MSG (19/1 ratio) at cell/protective agent ratios of 1/20 and 1/3 (w/w), mixed well and spray dried.

2.2.4. Effect of culture media

New sub-cultured inoculum was transferred to MRS or M7 broth medium with the following compositions in g/L: $MgSO_4.7H_2O$ 2.58; $K_2HPO_4.2H_2O$ 6.08; Glucose 12.435; Yeast Extract 55. Cells were harvested by centrifugation and were re-suspended into SM+MSG (19/1 ratio) (Table 1) at a cell/protective agent ratio of 1/3 (w/w), mixed well and spray dried.

2.2.5. Effect of stress adaption

Heat stress: Prepared culture grown in MRS medium was re-suspended in SM (Table 1) at a cell/protective agent ratio of 1/3 (w/w) with subsequent heat treatment in a water bath at 45, 50, 55 °C for 15 minutes, the control sample was kept at 30 °C.

Osmotic stress: New sub-cultured inoculum was transferred to MRS medium supplemented with either 5 % NaCl or 12 % NaCl, or 12 % NaCl with 1 % Monosodium glutamate. After three days, cells were harvested by centrifugation and re-suspended in SM solution (Table 1) at a cell/protective agent ratio of 1/3 (w/w).

2.2.6. Survivability of bacteria

All spray dried cultures were determined for their survivability right away after spraying or after storage using bacterial count in MRS - Agar supplemented with 5 % NaCl. Enumeration of

the bacteria was performed in triplicate after incubation for three days at 35 °C and the total counts of the viable bacteria were expressed as log colony forming units per gram (CFU/g). The results were expressed as the mean ± standard deviation from triplicate samples in each of at least two independent experiments.

Survival rate: $$S(\%) = \frac{N_a}{N_0} \times 100$$

N_0 : number of viable bacteria in 1 g of the preparation before drying or storage (CFU/gdb)

N_a : number of viable bacteria in 1 g of the obtained preparation after spray drying or storage (CFU/gdb)

2.2.7. Statistical analysis

The factors influencing the survival rate of *Tetragenococcus halophilus* CH6-2 in spray drying process were evaluated by ANOVA and Tukey test ($P < 0.05$) for comparison.

3. RESULTS AND DISCUSSION

3.1. Effect of protective agents

Various protective agents gave different survival rates (Figure 1A). After spray drying, the highest survival rate was observed for SM+MSG (10/10) system, reaching 0.182 %, while the lowest was recorded for MD, which was less than 0.01 %. Systems containing SM usually gave higher survival rates, as observed in SM+MSG+S, SM+MSG, SM+S, and SM systems (ranging from 0.077 to 0.182 %).

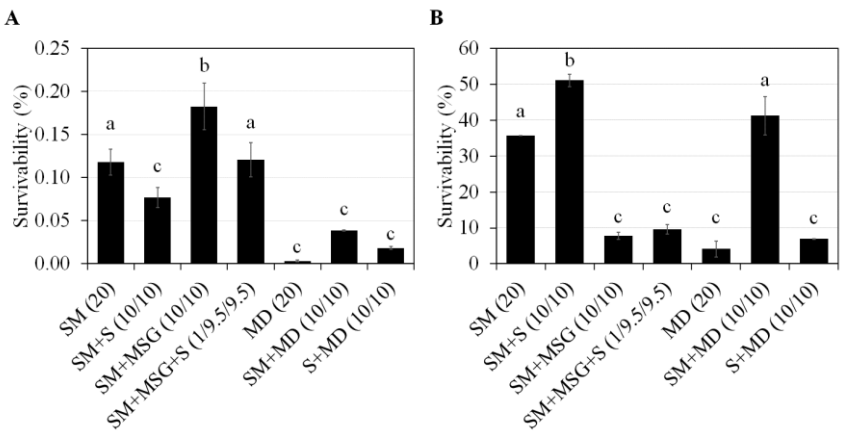

Figure 1. Survivability of *T. halophilus* CH6-2 after spray drying using different protective agents (A); after storage for three months (B). Different letters indicate a significant difference between bacteria populations ($P < 0.05$). Error bars represent SD (n = 3).

Huang suggested that Ca^{2+} may stabilize the cytoplasmic membrane under heat stress, and combine with milk protein to form a protective coating on bacteria cell walls [3]. The system containing SM (in SM+MD) showed a higher survivability than S+MD or MD alone. However, with the addition of MD in those systems, the survivability was consistently lower ($P < 0.05$) (varying from 0.01 to 0.033 %). Soukoulis (2013) demonstrated that MD, either alone or in

combination with other protective agents, always reduced the survivability of *L. acidophilus* NCIMB 701748 compared to milk protein after spray drying [9]. MSG seemed to have a positive effect on survivability as the two highest survival values were recorded for MSG systems ($P < 0.05$). Glutamate was revealed to be a precursor molecule for proline, which supports cells to withdraw the high salinity and therefore could be beneficial for cells to survive during spray drying [10].

The presence of a carbohydrate group had a positive effect on *T. halophilus* CH6-2 cells during storage as the highest survivability of *T. halophilus* CH6-2 was recorded for SM+S and SM+MD (Figure 1B) ($P < 0.05$). The survival rate in SM systems only decreased 1.2 and 1.4 times compared with SM+S, SM+MD but increased from 3.7 to 8.6 times compared with the rest systems. The protection of MSG during storage was less effective than that of S and MD. Overall, viable cells expressed as log CFU/g in dried powder after three months of storage were highest in the SM+MSG and SM+S systems.

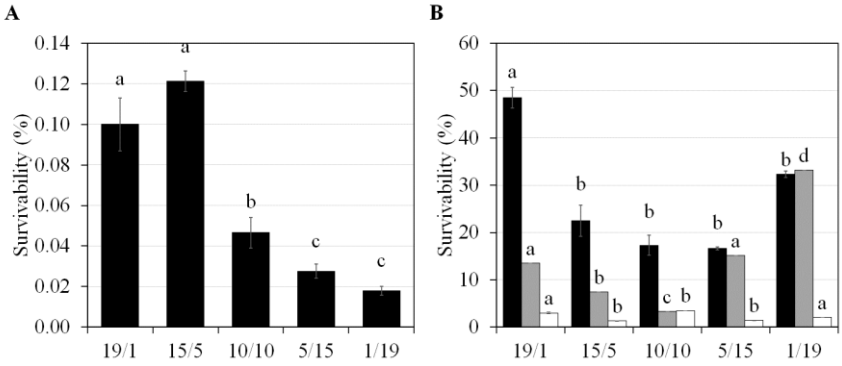

Figure 2. Survivability of *T. halophilus* CH6-2 using different ratios of SM+MSG after spray drying (A); after storage (B) for one month (black bar), two months (white bar), three months (grey bar). Different letters indicate a significant difference between bacteria populations ($P < 0.05$). Error bars represent SD ($n = 3$).

The use of MSG as a protective agent for formulation of *T. halophilus* CH6-2 dried preparation is desired since MSG is associated with umami taste in fish sauce and thus does not affect its sensory characteristics. In the next experiment, SM and MSG were added at various ratios to obtain different systems (Table 1). The highest survivability after spray drying was recorded for SM+MSG with the ratios of 19/1 and 15/5 ($P > 0.05$), the lowest at the ratio of 1/19 ($P < 0.05$). Increasing the MSG ratio in the system reduced the survivability of *T. halophilus* CH6-2 strain after spray drying. A similar result was also reported by Golowczyc (2011) for *Lactobacillus kefir* CIDCA 8321 and *L. kefir* CIDCA 8348 [11]. Survival rate was high at high concentrations of SM or MSG during two months of storage but was quickly reduced after 3 months to similarly low level at all ratios. Golowczyc demonstrated that adding MSG to the SM system reduced the viability of *L. kefir* CIDCA 8321 marginally compared to the SM alone [11]. Overall, the viable cell in dried powder after three months of storage was the highest in the SM+MSG systems with a ratio of 19/1. Our results indicated that high MSG concentrations affected the survivability of *T. halophilus* CH6-2.

Another factor to consider is the powder recovery yield. Table 2 shows that the SM+MSG system with the ratio of 19/1 had the highest recovery yield. As the MSG ratio increased, the recovery yield was reduced. This was due to low glass transition temperature of MSG [3]. This

result emphasized the advantages of using SM+MSG at the ratio of 19/1 as a protective agent for spray drying of *T. halophilus* CH6-2.

Table 2. Powder recovery yield (%) using different ratios of SM and MSG.

Protective agents	Concentration (% w/v)	Powder yield (%)	Drying temperature
SM+MSG	19+1	50.67	84 ± 3 ℃
	15+5	47.62	
	10+10	49.05	
	5+15	35.14	
	1+19	31.14	

3.2. Effect of cell/protective agent ratio

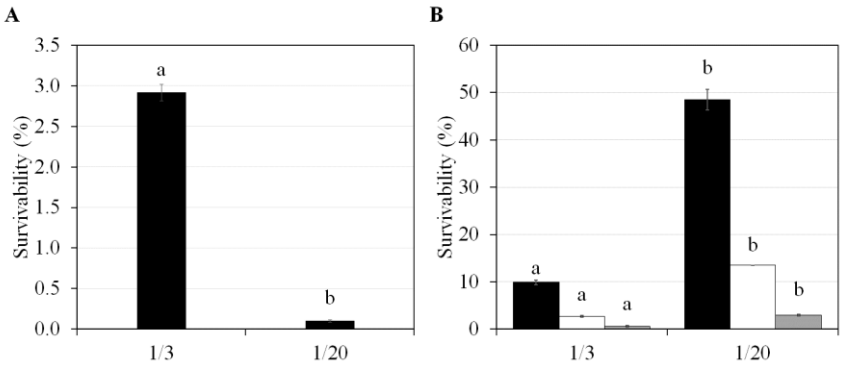

Figure 3. Survivability of *T. halophilus* CH6-2 with different ratios of cell/protective agents after spray drying (A); after storage (B) for one month (black bar), two months (white bar), three months (grey bar). Different letters indicate a significant difference between bacteria populations ($P < 0.05$). Error bars represent SD ($n = 3$).

To increase viable cells in the dried preparation, a higher initial cell concentration through a higher cell/protective agent ratio was applied. In Figure 3A, the sample with a cell/protective agent ratio of 1/3 gave a higher survival rate ($P < 0.05$), reaching 2.91 %, which was 29 times higher than that of the sample with a ratio of 1/20. The viable cells in the dried preparation increased from 6.63 log CFU/g to 10.573 log CFU/g (Table 3).

Table 3. Viable cell count (log CFU/g) with different ratios of cell/protective agents.

Ratio of cell/protective agents		Log viable cell count (log CFU/g)		
	Before spray drying	After spray drying	After 1 month of storage	After 3 months of storages
1/20	9.630	6.630	6.315	5.106
1/3	12.107	10.573	9.568	8.368

The result was in constrast to an observation by Broeckx, who reported that increasing the cell/protecting agent ratio from 1/4 (initial cell concentration of 10.97 log CFU/g) to 4/1 (initial cell concentration of 11.43 log CFU/g) reduced the survivability [5]. Meanwhile, Palmfeldt found that only initial cell concentration from 10^9 to 10^{10} gave high survival value after freeze

drying, outside this range the value dropped almost ten times [12]. There are only few publications on the optimal initial cell concentration for spray drying. The optimal initial cell concentration was found to be related to the protective agent used [12].

After three months of storage, the survival rate of *T. halophilus* CH6-2 in the sample with a ratio of 1/20 was 4.9 times higher than in the sample with a ratio of 1/3 (Figure 3B) ($P < 0.05$), however the actual viable cell in the 1/3 sample after storage was much higher (Table 3). In addition to the lower viable cells in the preparation, the high protective agent ratio of 1/20 might affect the sensory quality of the product. Therefore, a cell-to-protective agent ratio of 1/3 seemed to be the better choice in this case. Further study is needed to find the optimal cell/protective agent ratio for enhancing the survival rate.

3.3. Effect of culture media

M7 was the optimized medium for *T. halophilus* CH6-2, which could double the cell concentration (our results) and thus higher viable cells could be expected in the dried powder. With the same protective agent, the survivability of *T. halophilus* CH6-2 cultured in M7 medium was 3.04 % after drying, slightly higher than in MRS medium (2.91 %) (Figure 4A) ($P > 0.05$). However, during storage, the survivability of *T. halophilus* CH6-2 cultured in MRS medium was about 6 to 8 times higher than that of M7 after one to three months of storage ($P < 0.05$). The main cause may come from the synthesis of compatible solutes in LAB depending on the presence of their precursors in the medium [10]. Parlindungan proved that the presence of Tween 80 (presented in MRS medium) increased the survivability of *L. plantarum* B21 [6].

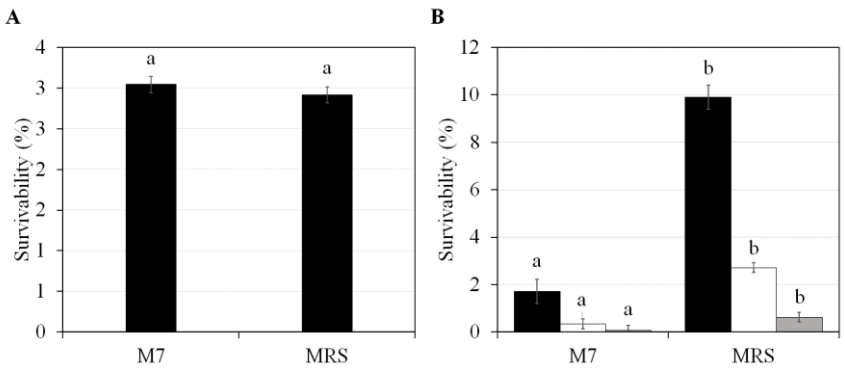

Figure 4. Survivability of *T. halophilus* CH6-2 grown in different media after spray drying (A); after storage (B) for one month (black bar), two months (white bar), and three months (grey bar). Different letters indicate a significant difference between bacteria populations ($P < 0.05$). Error bars represent SD ($n = 3$).

3.4. Effect of stress adaption

Heat stress adaption has been widely used to increase the tolerance of bacteria to spray drying. However, our results showed a negative effect of heat stress, as the higher the temperature of heat-treating cells, the lower the survivability of *T. halophilus* CH6-2 after spray drying (Figure 5A). It can be explained that heat treatment caused damage to cells, leading to a reduction in survivability [13]. Desmond (2001) observed a similar phenomenon that heat stress reduced the survivability of *Lactobacilli* when T_{outlet} was below 95 °C [8].

Similar results were obtained for storage, that is, heat stress adaption affected the survivability, leading to a lower survival rate. However, the higher survivability observed for cells heat-treated at 55 °C after storage than at a lower temperature of 45 - 50 °C promised the potential of stress adaption. The temperature and time applied in this study did not appear to be sufficient to trigger the stress adaption of *T. halophilus* CH6-2 ($P > 0.05$). *T. halophilus* regularly faces stresses including osmotic stress (high salt concentration), low pH stress during food fermentation and may need harder stress adaption conditions to improve its survivability.

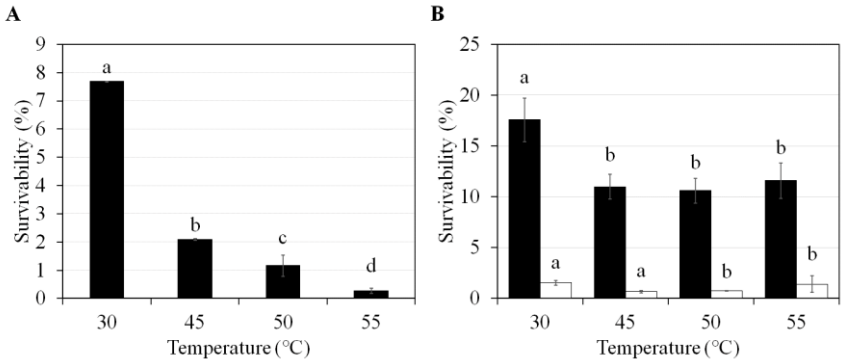

Figure 5. Survivability of *T.halophilus* CH6-2 after spray drying (A); after storage (B) for one month (black bar), three months (white bar). Different letters indicate a significant difference between bacteria populations ($P < 0.05$). Error bars represent SD (n = 3).

Similar to heat stress, osmotic stress did not improve the survivability of *T. halophilus* CH6-2 during both spray drying and storage (Figure 6). The addition of MSG neither improved the survivability. There have been only a few studies on osmotic adaption before spray drying. Osmotic stress was also reported to bring no increase to the survivability of *L. plantarum* [14]. However, the survivability of *T. halophilus* CH6-2 with the addition of 1 % MSG after two months of storage reached 29.2 %, higher than the cells without the MSG addition ($P < 0.05$). Higher survivability of *Lactobacillus sakei* cells grown on MSG-supplemented medium was also observed [15]. The result suggested that the addition of MSG to the growth medium could improve the survivability of *T. halophilus* CH6-2 during storage. Further study is needed to investigate to confirm this observation.

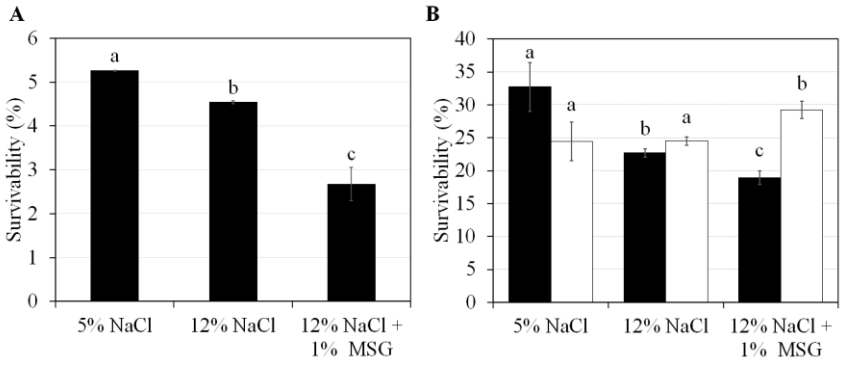

Figure 6. Survivability of *T. halophilus* CH6-2 with osmotic treatments after spray drying (A); after storage (B) for one month (black bar), two months (white bar). Different letters indicate a significant difference between bacteria populations ($P < 0.05$). Error bars represent SD (n = 3).

From our study, the survival rate after spray drying of *T. halophilus* CH6-2 reached the highest (7.7 %) when cells were cultured in MRS medium supplemented with 5 % NaCl and protective agent skim milk at a cell/protective agent ratio of 1/3 without heat or osmotic stress (Figure 5A). The survival rate for lactic acid bacteria depends strongly on strain type, outlet temperature, protective agent, stress adaption and many other parameters. The survival rate for *L. acidophilus* NCIMB 701748 was reported to range from 0.44 % to 69.9 % [9], for *L. paracasei* NFBC 338 from 0.5 % to 33.46 % [8], and for *L. rhamnosus* GG from 4 % to 50 % [5].

4. CONCLUSION

The study showed that SM had a positive effect during spray drying and storage on *T. halophilus* CH 6-2. Low concentrations of MSG in SM+MSG system could protect *T. halophilus* CH6-2 cell during spray drying, while high MSG concentrations reduced the survivability during both spray drying and storage. MRS was the better medium than M7 for culturing *T. halophilus* CH6-2 in respect of survivability after storage. Increasing cell/protective agent ratio from 1/20 to 1/3 could increase the survival rate after spray drying but not during storage, giving significantly higher viable cells in dried preparation after spray drying and storage. Heat and osmotic stress adaption conditions used in this study did not have a clear effect on improving the survivability of *T. halophilus* CH6-2 after both spray drying and storage.

Acknowledgments. This work was funded by the Ministry of Education and Training through project B2021-BKA-18.

Authors contributions. Nguyen Trung Thanh performed experiments, prepared Tables and Figures, contributed to the final manuscript. Vu Thi Kieu Oanh performed experiments. Le Thanh Ha conceived and designed the experiments, discussed the results, and approved the final manuscript.

Conflict statement. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Udomsil N., Rodtong S., Choi Y. J., Hua Y., and Yongsawatdigul J. -Use of Tetragenococcus halophilus as a starter culture for flavor improvement in fish sauce fermentation, J. Agric. Food Chem. **59** (15) (2011) 8401-8. doi: 10.1021/jf201953v.
2. Ha L., Trinh N., HauL. C, and Tu N. - Characterization of Tetragenococcus halophilus from Vietnamese fish sauce, Vietnam Journal of Science and Technology **57** (5) (2019) 544-550. doi: 10.15625/2525-2518/57/5/13272.
3. Huang S., Vignolles M. L., Chen X. D., Loir Y. L., Jan G., Schuck P., and Jeantet R. - Spray drying of probiotics and other food-grade bacteria: A review, Trends in Food Science & Technology **63** (2017) 1-17. doi: 10.1016/j.tifs.2017.02.007.
4. Peighambardoust S. H., Golshan Tafti A., and Hesari J. - Application of spray drying for preservation of lactic acid starter cultures: a review, Trends in Food Science & Technology **22** (5) (2011) 215-224.doi: 10.1016/j.tifs.2011.01.009.
5. G. Broeckx, Kiekens S., Jokicevic K., Byl E., Henkens T., Vandenheuvel D., Lebeer S., and Kiekens F. - Effects of initial cell concentration, growth phase, and process parameters on the viability of Lactobacillus rhamnosus GG after spray drying, Drying Technology **38** (11) (2019)1474-1492. doi: 10.1080/07373937.2019.1648290.

6. Parlindungan E., Dekiwadia C., May B. K., and Jones O. A. H. - Nutrient stress as a means to enhance the storage stability of spray dried microencapsulated Lactobacillus plantarum B21, Lwt **111** (2019) 204-210. doi: 10.1016/j.lwt.2019.05.010.
7. He G., Deng J., Wu C., and Huang J. - A partial proteome reference map of Tetragenococcus halophilus and comparative proteomic and physiological analysis under salt stress, RSC Advances **7** (21) (2017)12753-12763. doi: 10.1039/c6ra22521g.
8. Desmond C., Stanton C., Fitzgerald G. F., Collins K., and Paul Ross R. - Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying, International Dairy Journal **11** (10) (2001) 801-808. doi:https://doi.org/10.1016/S0958-6946(01)00121-2.
9. Soukoulis C., Behboudi-Jobbehdar S., Yonekura L., Parmenter C., and Fisk I. - Impact of Milk Protein Type on the Viability and Storage Stability of Microencapsulated Lactobacillus acidophilus NCIMB 701748 Using Spray Drying, Food and Bioprocess Technology **7** (5) (2013) 1255-1268. doi: 10.1007/s11947-013-1120-x.
10. Saum S. H. and Muller V. - Salinity-dependent switching of osmolyte strategies in a moderately halophilic bacterium: glutamate induces proline biosynthesis in Halobacillus halophilus, J. Bacteriol **189** (19) (2007) 6968-75. doi: 10.1128/JB.00775-07.
11. Golowczyc M. A., Gerez C. L., Silva J., Abraham A. G., De Antoni G. L., and Teixeira P. - Survival of spray-dried Lactobacillus kefir is affected by different protectants and storage conditions, Biotechnol Lett **33** (4) (2011) 681-6. doi: 10.1007/s10529-010-0491-6.
12. Palmfeldt J., Rådström P., and Hahn-Hägerdal B. - Optimisation of initial cell concentration enhances freeze-drying tolerance of Pseudomonas chlororaphis, Cryobiology **47** (1) (2003) 21-29. doi: 10.1016/s0011-2240(03)00065-8.
13. Salar-Behzadi S., Wu S.,Toegel S., Hofrichter M., Altenburger I., Unger F. M., Wirth M., and Viernstein H. - Impact of heat treatment and spray drying on cellular properties and culturability of Bifidobacterium bifidum BB-12, Food Research International **54** (1) (2013) 93-101. doi: 10.1016/j.foodres.2013.05.024.
14. Bustos P. and Bórquez R. - Influence of Osmotic Stress and Encapsulating Materials on the Stability of Autochthonous Lactobacillus plantarum after Spray Drying, Drying Technology **31** (1) (2013) 57-66. doi: 10.1080/07373937.2012.717325.
15. Ferreira V., Soares V., Santos C., SilvaJ., Gibbs P. A., and Teixeira P. - Survival of Lactobacillus sakei during heating, drying and storage in the dried state when growth has occurred in the presence of sucrose or monosodium glutamate, Biotechnology Letters **27** (4) (2005) 249-252.doi: 10.1007/s10529-004-8351-x.