LWT - Food Science and Technology 77 (2017) 152–159

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

# A novel soybean flour as a cryoprotectant in freeze-dried *Bacillus subtilis* SB-MYP-1

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ARTICLE INFO

*Article history:*
Received 6 June 2016
Received in revised form
13 October 2016
Accepted 6 November 2016
Available online 9 November 2016

*Keywords:*
Freeze drying stress
Soybean flour
Cryoprotectant
Encapsulation
*Bacillus subtilis*

*Chemical compounds studied in this article:*
Sodium chloride (Pubchem CID: 5234)
Glutaraldehyde (Pubchem CID: 3485)
Dipotassium hydrogen phosphate (Pubchem CID: 24450)
Potassium dihydrogen phosphate (Pubchem CID: 516951)
1,6-Diphenyl-1,3,5-hexatriene (Pubchem CID: 5376733)
Methanol (Pubchem CID: 887)
Potassium chloride (Pubchem CID: 4873)
Calcium dichloride (Pubchem CID: 5284359)
2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (Pubchem CID: 64957)
Tris hydrochloride (Pubchem CID: 93573)
NAD (Pubchem CID: 5892)
Glutamic acid (Pubchem CID: 33032)
2-Mercaptoethanol (Pubchem CID: 1567)
EDTA (Pubchem CID: 6049)
2-Oxoglutaric acid (Pubchem CID: 51)
L-glutamine (Pubchem CID: 5961)
Magnesium sulphate (Pubchem CID: 24083)
HydroxyLamine (Pubchem CID: 787)
ATP (Pubchem CID: 13803)
Trichloroacetic acid (Pubchem CID: 6421)
Ethidium bromide (Pubchem CID: 14710)

ABSTRACT

*Bacillus subtilis* SB-MYP-1 isolated from Thai Thua-nao fermentation has already been demonstrated as a potential starter culture. The aim of this work was to reveal the efficiency order of soy protein isolate (SPI), soluble starch (ST), maltodextrin (MD), and Doikham soybean flour (SBF) for protecting *B. subtilis* SB-MYP-1 cells under sub-lethal stress of freeze-dried encapsulation. The combination criteria, including the survival rate, low water activity, and moisture content of freeze-dried cells with SBF, were approximately 63.72% ($1.30 \times 10^8$ CFU/g as cell viability), 0.285, and 2.31% wet basis, respectively, which were relative to the standard quality of powdered starter culture. In addition, the cell membrane fluidity, metabolic retardation, activities of three intracellular enzymes (glutamate dehydrogenase, GDH; 2-oxoglutarate aminotransferase, GOGAT; and glutamine synthetase, GS), and *pgsB* gene expression of freeze-dried cells were maintained by SBF protection. Surprisingly, the mode of action of SBF could preserve the protein stabilization, phospholipid bilayer, and peptidoglycan surface in freeze-dried cells, whereas the partial cell surface and lipid bilayer of cells with SPI, ST and MD were destroyed after sub-lethal freeze drying. This study revealed the novel application of SBF as a cryoprotective agent for further freeze-dried *B. subtilis* SB-MYP-1 preservation.



## 1. Introduction

Freeze-dried bacteria, yeast, and mould have been commonly used as starter cultures in numerous fermented food industries

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http://dx.doi.org/10.1016/j.lwt.2016.11.015
0023-6438/

because the freeze drying technique can control significant quality parameters, including the moisture content, water activity ($a_w$), and cell viability, based on the standard guidelines of ATCC and NCTC (Morgan, Herman, White, & Vesey, 2006). Moreover, the major problems of freeze-dried stress affect the sub-lethal conditions of cell targets, such as decreasing the cell viability, cell membrane fluidity and intracellular enzyme activity, while gene damage remains (Cavicchioli, Saunders, & Thomas, 2014; Li et al., 2011; Morichi & Irie, 1973; Wang, Yu, Xu, Aguilar, & Wei, 2016). Similarly, Li et al. (2011) reported that cold stress could directly damage the cell membrane fluidity, protein, and DNA function of *Lactobacillus reuteri* cells because of intracellular-ice crystallization in the presence of freeze-drying. Likewise, the cell wall of *L. helveticus* was broken after low vacuum at 100 mbar (Santivarangkna, Wenning, Foerst, & Kulozik, 2007). In addition, the low pressure induced low moisture stress, decreasing the intracellular enzyme activities of dried *L. reuteri* (Li et al., 2011).

For freeze-dried processing of a starter culture, it is generally recommended to use commercial cryoprotectants, such as soy protein isolate, soluble starch, maltodextrin and its derivatives, to protect against freeze drying injuries, but those agents have high cost. A good cryoprotectant should have a low cost and the ability to be dried to the microbial cells during the freeze drying process. Additionally, it should also provide a good matrix for rehydration and stabilization. A suitable cryoprotectant was, therefore, considered for the freeze-dried starter culture, which was inoculated during food fermentation and did not affect the fermented food properties (Burgain, Gaiani, Linder, & Scher, 2011; Dianawati, Mishra, & Shah, 2013; Mahidsanan & Gasaluck, 2011; Pyar & Peh, 2011; Rajam & Anandharamakrishnan, 2015; Ray, Raychaudhuri, & Chakraborty, 2016; Santivarangkna, Higl, & Foerst, 2008).

The starter culture used in this study was *Bacillus subtilis* SB-MYP-1, isolated from fermented soybean (named Thua-nao in Thai), which significantly synthesizes the nutritional metabolite poly-γ-glutamic acid (PGA) during fermentation due to the consequence of *pgsB* gene expression and three intracellular enzyme (GDH, GOGAT, and GS) activities (Mahidsanan & Gasaluck, 2011; Najar & Das, 2015; Ruzal & Sanchez-Rivas, 2003; Shih & Van, 2001; Shih, Wu, & Shieh, 2005; Zeng et al., 2014). Gasaluck (2010) confirmed that the advantages of this starter culture were substantiated in Thua-nao fermentation, whose product properties consisted of the good pyrazine compound, nutritional values (calcium, ferric, phosphorus, and vitamin B12), and safety. *B. subtilis* SB-MYP-1 is, therefore, a beneficial starter culture to be preserved with the appropriate cryoprotectant.

Soybean flour (SBF), a Doikham product from The Royal Project Foundation, was an alternative cryoprotectant that consisted of protein (34.20 g/100 g), carbohydrate (36.00 g/100 g), and lipid (21.10 g/100 g) because the cost is lower than that of commercial cryoprotectants (Gasaluck, 2015). Freeze-dried *B. subtilis* SB-MYP-1 encapsulated with SBF was initially reported by Gasaluck (2015). It was suggested that this dried culture could be directly inoculated into Thua-nao supplemented with PGA fermentation, and the organoleptic properties of the fermented product were not affected.

At present, there is no report of freeze-dried *B. subtilis* using the SBF mechanism. The main purposes of this study were, therefore, to validate an innovative SBF cryoprotectant for preventing *B. subtilis* SB-MYP-1 cell damage after freeze drying stress. Additionally, its viability, cell membrane fluidity, morphological properties, metabolic activity, activities of three intracellular enzymes and *pgsB* gene expression were compared with those of three different commercial cryoprotectants.

## 2. Materials and methods

### 2.1. Bacterial culture

*B. subtilis* SB-MYP-1 was obtained by isolation from fermented soybean (Thua-nao). *B. subtilis* biochemical identification was confirmed using API 50 CHB medium (Bio Merieux Inc), and a sample was kept as a certified stock at the School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. Its cell suspension was prepared by transferring 0.5 mL of stock culture to 10 mL of nutrient broth followed by incubation in a shaker at 200 rpm and 37 °C for 24 h. Cells were harvested by centrifugation (10 000 × g for 10 min at 4 °C) and washed twice with 0.85% (w/v) sterile sodium chloride solution. The population of *B. subtilis* SB-MYP-1 in the suspension was approximately $10^8$–$10^9$ CFU/mL.

### 2.2. Freeze drying method

The 10% (w/v) cell pellet was mixed with equal proportions of either sterile 10% (w/v) soy protein isolate (SPI; Chemipan product), 10% (w/v) soluble starch (ST; Carlo Erba product), 10% (w/v) maltodextrin (MD; Chemipan product) or 10% (w/v) soybean flour (SBF; Doikham Food Product Co. Ltd, Thailand) to reach a final of cell concentration of $10^8$–$10^9$ CFU/mL. Those suspensions were frozen at −60 °C overnight and then desiccated under a vacuum (0.001 mbar) in the freeze-drier (Christ Gamma 2–16 LSC) at a final temperature of 35 °C for 24 h.

### 2.3. Enumeration

To quantify the viability of the cells, each sample was resuspended in an appropriate volume of 0.85% (w/v) sterile sodium chloride solution. After appropriate serial dilutions, the samples (0.1 mL) were spread on plate count agar and incubated at 37 °C for 24 h. The survival rate for each sample tested was expressed as $(N/N_0) \times 100$, where $N_0$ and N were the numbers of viable cells before and after freeze drying, respectively. All enumerations were performed in duplicate, and the plates containing 30–300 colonies were counted and calculated as CFU/g of freeze-dried culture or mL of solution.

### 2.4. Moisture content and water activity measurement

The moisture content of freeze-dried culture at the end of the freeze drying process was determined by the AOAC method (AOAC., 2000), and the water activity ($a_w$) was measured using an Aqua Lab CX-2 instrument at room temperature.

### 2.5. Morphological characterization

A fresh culture of *B. subtilis* SB-MYP-1 was washed twice with 0.1 M potassium phosphate buffer (pH 6.5) and immersed in glutaraldehyde (1 g/100 mL) at 4 °C for 2 h. The fixative was removed by washing three times with 0.1 M potassium phosphate buffer, and the cells were subjected to dehydration with an increasing concentration of ethanol (0, 50, 70, 80, 90, 95 and 100 mL/100 mL) for 10 min. After complete evaporation of ethanol, the resulting sample was dried at 50 °C overnight and then simultaneously mounted with all freeze-dried samples on carbon stumps and coated with a gold I sputtering device (JFC-1100E). The morphology of each sample was analysed by scanning electron