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Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying

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ABSTRACT

Stabilization efficiency in terms of long term ambient temperature storage viability of the probiotic strain *Lactobacillus casei* CRL 431 was compared using freeze and fluidized bed drying techniques. Fluidized bed drying was able to retain 2.5 log cfu/g higher viability after 52 weeks of storage at 25 °C. A combination of fluidized bed drying and osmotic stress adaptation to the probiotic cells yielded further improvement of 0.83 log cfu/g higher viability compared to the unstressed cells. The findings were validated with other two lactobacilli and two bifidobacterium strains with probiotic characteristics and significant improvements in storage stability over freeze dried samples were observed. Fortification of vitamin E in the stabilization matrix as an antioxidant improved the stability by 0.18 log cfu/g during 20 weeks storage period at 25 °C, whereas any similar benefit of fortifying inulin as a prebiotic was not observed.

1. Introduction

Probiotic containing food products are major contributors to the continuously growing functional food markets around the world. Probiotics are generally found in fermented dairy products like fermented milk, yoghurts and cheese, among others, and in dried forms dietary supplements and infant formula are the most common vehicles to offer them to the consumers. A very important health benefit from consuming probiotic bacteria, namely antimicrobial activities against pathogens have recently been validated (Rodriguez et al., 2012). Probiotic cultures are stabilized and fortified into powdered food or neutraceutical formulations. At the end of their shelf life (typically 6 months to 1 year), the product need to maintain a cell viability of at least 10^6 colony forming units (cfu) per g to confer the desired health benefits (FAO/WHO, 2003). In dried products stored in ambient environment, a significant reduction of probiotic viability during the storage can cause considerable reduction in the product shelf life or may cause serious violation of the regulatory norms. The existence of this challenge of maintaining adequate probiotic viability is clearly evident from the labelling claims of many products available in the market which disclose the viable probiotic content per g 'at the time of manufacturing' but do not ensure that the same level would be available at any point during their claimed shelf life. It has been reported that the cell survival during storage in a dry form is mostly affected by an elevated water activity ($a_w > 0.25$) (Teixeira, Castro, Malcata, & Kirby, 1995), storage temperature and presence of atmospheric oxygen (Anal & Singh, 2007). The particular need of maintaining probiotic viability at a relatively higher storage temperature is highly important considering the lack of proper cold chain distribution system throughout the developing countries.

Freeze drying is the most popular stabilizing technique for probiotics because of the milder heat processing involved.

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However, the detrimental effects of freeze drying have also been reported as undesirable changes in the physical state of cell membrane lipids and the structures of sensitive cell proteins (Leslie, Israeli, Lighthart, Crowe, & Crowe, 1995). In addition, the process is expensive and therefore the common end products in the form of dietary supplements are inaccessible to the underprivileged population of the world. The use of fluidized bed drying technique in probiotic stabilization has not been extensively explored and only a handful of studies could be found where the fluidized bed has been used as a spray coater (Haris, Ramasamy, Julianto, Sieo, & Ho, 2012; Kumar et al., 2010; Stummer et al., 2012). In the fluidized bed dryer, the probiotic cell suspension is mixed with a vibrating bed of absorbers or matrix molecules which helps to form the capsules by adherence (Champagne & Fustier, 2007). A simultaneous supply of moderately hot air flow from the bottom is provided to dry off the aqueous medium and bring down the powdered matrix to a desired water activity level. This process is comparatively economic, involves low energy consumption and imparts moderate heat stress to the bacterial cells but with the limitation of being a batch process (George, Cenkowoski, & Muir, 2004; Kudra & Mujumdar, 1988).

This contribution discusses the stabilization of a probiotic strain *Lactobacillus casei* CRL 431 with both freeze and fluidized bed drying and compares the residual viability over a period of 52 weeks when stored at 25 °C. Then we moved onto investigating the effects of heat, osmotic and combined stress adaptations on long term ambient stability of this strain. The best combination of drying technique and stress adaptation which showed promising results during storage at 25 °C were selected and further experiments were conducted to enhance the stability by the incorporation of an antioxidant and a prebiotic compound in the stabilization matrix. Finally, we wanted to validate our findings over a range of other popular probiotic strains and therefore applied the best known technique to another two lactobacilli and two bifidobacterium strains. We compared their ambient stability with the freeze dried samples of the same strains when stored at 25 °C.

2. Materials and methods

2.1. Preparation of bacterial cell suspension

In this study, altogether three lactobacilli and two bifidobacterium strains were used. The lactobacilli strains were *L. casei* CRL 431 (ATCC Accession No. 55544), *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 53103 sourced through Cryosite Ltd. (Lane Cove, NSW, Australia) and the bifidobacterium strains were *Bifidobacterium lactis* BB12 from Chr. Hansen (Horsholm, Denmark) and *B. lactis* HN019 from Danisco (Braband, Denmark). The freeze dried cultures were rehydrated in MRS broth from Difco Lab (Franklin Lakes, NJ, USA) at 37 °C for 24 h followed by two consecutive growth cycles up to their early stationary phase. The growth phases of individual strains, especially the early log, mid log and early stationary phases were identified by the standard optical density measurement process at 610 nm. Cells were harvested by centrifugation at 4600*g* for 10 min and washed two times with 0.25% peptone water (Nag, Han, & Singh, 2011).

2.2. Stress adaptation process

The initial set of experiments were conducted using only *L. casei* CRL431 cells by applying heat, osmotic, a combination of heat and osmotic stresses and compared with unstressed cells as control. We wanted to develop the methods and techniques with one strain first and then move onto other strains to validate the findings, hence the choice of *L. casei* CRL 431 was random. A slightly modified experimental design based on the works of Pichereau, Hartke, and Auffray (2000) and Prasad, McJarrow, and Gopal (2003) was planned. According to the growth curve obtained for this strain, 14th and 20th hours were identified as the mid log and early stationary phases, respectively. For the heat stress adaptation, after 20 h of growth, the cells suspended in the growth media were subjected to gradual heating in a water bath maintained at 50 °C and held for 30 min. Thereafter the suspension was immediately cooled down to room temperature (22 °C) and cells were harvested as mentioned in the Section 2.1. The osmotic stress to the cells was induced at the mid log phase (14th hour) by addition of required quantity of NaCl to give a final osmolality of the media as 0.6 M of NaCl. Incubation at 37 °C was continued till the early stationary phase and the same harvesting procedure as mentioned above was followed. For a combined heat and osmotic stress adaptation, cells were subjected to the osmotic stress first as described above and then were harvested by centrifugation. The obtained pellet was re-suspended into fresh MRS broth and the same heat stress (50 °C/30 min) was applied thereafter.

2.3. The stabilization process

For freeze drying process, washed cell pellets were added to 20% (w/w) pre-sterilized reconstituted whole milk. The mix was homogenized in an Ultra-Turax mixer from Ika Works (Guangzhou, China) at 9000 rpm and freeze dried in a dryer (Model 0610) from W.G.G. Cuddon Ltd. (Blenheim, New Zealand). The steps involved in the process were freezing at −18 °C, followed by sublimation at 20 °C under vacuum of 0.4 Torr for a 48 h period. The dried flakes were manually ground with a mortar and repacked in double layered LDPE sachets. In case of fluidized bed drying (FBD) a modified protocol suggested by Haris et al. (2012) and Stummer et al. (2012) was followed. Washed cell pellets were first mixed homogeneously with whole milk powder from Fonterra Co-operative (Palmerston North, New Zealand) containing approximately 26% fat. The ratio of cell pellet to milk powder was determined based on a final desired water activity of 0.45–0.50, which have been found easy to handle and dry in the FBD. In one of the experiments, effects of vitamin E as an antioxidant and inulin as a prebiotic compound were evaluated by incorporating them into the stabilization matrix. Vitamin E (50% dl-alpha-tocopheryl acetate) powder from DSM Nutritional Products Ltd. (Wurmisweg, Switzerland) at 0.5% (w/w) and inulin from Sensus Operations CV (Roosendal, The Netherlands) at 5.0% (w/w) of the final product, both of them were mixed directly to the centrifuged cell pellet so that the bacterial cells get maximum contact and hence the best possible synergistic effects from them. The cell suspension and whole milk powder mix was then transferred to a lab scale FBD from

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