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Effect of different immobilization media on breakdown of whey proteins by *Streptococcus thermophilus*

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ABSTRACT

Objective: In this study, we aimed to compare the efficiency of different immobilization media to facilitate breakdown of whey proteins by *Streptococcus thermophilus (S. thermophilus)*.

Materials and Methods: *S. thermophilus* was isolated from yoghurt. High-protein whey powder was present in fermentation media and two-phase dispersion technique was used for immobilization of *S. thermophilus* in agar, agarose and κ-carrageenan. Total protein after fermentation of whey proteins with *S. thermophilus* in different media was measured. We have also performed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis to observe changes in individual whey proteins after fermentation in different media.

Results: Total protein concentration showed a significant decrease at the end of 24 hours of fermentation in all media. SDS-PAGE results showed that the amount of both α-lactalbumin and β-lactoglobulin were reduced in all immobilization media compared to control. The effect of κ-carrageenan was considerably higher compared to other media.

Conclusion: Our results showed that immobilization in κ-carrageenan increased the breakdown of whey proteins by *S. thermophilus* and can be used to increase fermentation efficiency.

Keywords: α-lactalbumin, β-lactoglobulin, Fermentation, Immobilization, κ-carrageenan

1. INTRODUCTION

Milk is an excellent source of fat, proteins, minerals and vitamins [1]. Cow's milk consists of 90% water, 5% carbohydrates and 4–5% proteins. Milk proteins are divided into two classes (groups): soluble whey proteins and casein phosphoproteins. Soluble whey proteins comprise β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin and lactoferrin. In addition, a large number of other proteins are present at lower concentrations [2]. The main allergens in milk are caseins, β-lactoglobulin and α-lactalbumin. Reduction or elimination of milk allergens by effective methods and technologies is essential to help consumption of milk by individuals who are allergic to these ingredients.

Fermentation is a traditional food processing technology. Fermented foods exert a positive influence on human health. This is mainly due to the ability to release bioactive peptides from food proteins by microbial enzymatic hydrolysis. It has been shown that lactic acid bacteria possess a complex proteolytic system composed of proteinases, peptidases and transport proteins. During fermentation, hydrolysis of milk proteins by lactic acid bacteria may have important effects on milk digestibility and production of bioactive peptides. Moreover, proteolysis can destroy some epitopes and consequently decrease allergenicity. Dietary consumption of probiotics and fermented foods, i.e. yoghurt can alleviate some symptoms of atopy and reduce development of allergies through immune regulation [3].

Immobilized cells exhibit many advantages over floating cells in food processing [4]. Among these are maintenance of stable and active biocatalysts, reuse of biocatalysts, accelerated reaction rates, high volumetric productivity, improved process control, reduced susceptibility of cells to contamination, improved production efficiency and no possibility of cell wash-out [5]. Gel entrapment in natural polymers such as

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alginates and κ-carrageenans is favored by many workers for immobilizing living cells [6-10]. These mild, cheap and simple methods of entrapment are non-toxic and preserve the integrity of immobilized biocatalysts. The choice of the entrapment polymer depends on the conditions of the fermentation process [4]. Alginate and κ-carrageenan gels have been widely used with lactic acid bacteria [5,11-13]. In this study, we aimed to compare the efficiency of different immobilization media to facilitate breakdown of whey proteins by *S. thermophilus*.

2. MATERIALS and METHODS

S. thermophilus isolated from yoghurt was used. Home-made yoghurt was mixed with an equal amount of phosphate-buffered saline (PBS). After 72 hours of incubation on M17 agar, resulting colonies were identified as *S. thermophilus* (99%) *subsp. salivarius* (33%) [14]. High-protein whey powder (6%) was centrifuged at 3.900 x g, 4°C for 1 hour and supernatant was used in fermentation media (pH 6.5, 200 mL). Sterilization was performed using two different techniques: serial filtration was performed (5.0 µm, 0.45 µm and 0.22 µm in order) and filtered whey protein supernatant (WP-F) was kept at 4°C, whey supernatant was autoclaved at 121°C for 15 minutes to obtain whey protein (WP-A) that was also kept at 4°C.

***Immobilization*:** Two-phase dispersion technique was used to achieve immobilization in agar, agarose and κ-carrageenan [15].

Agar: 1 g agar was mixed with 50 mL ultra-pure H_2O and autoclaved at 121°C for 15 min. Polymer was cooled down to 50-55°C and then 100 µL of bacteria (1 x 10^7 cfu/mL) was added and dropped into the oil-phase serially (1 mL). Beads were left at oil-phase for 2.5 min for spherical shaping to be completed, washed with 0.01% Triton-X and put into the aqua-phase (ultra-pure water) and left for 5 min. Finally, beads were mixed with whey medium (5 mL).

Agarose: 1 g agarose was mixed with 50 mL ultra-pure H_2O and autoclaved at 121°C for 15 min. Polymer was cooled down to 40-45°C and then 100 µL of bacteria (1 x 10^7 cfu/mL) was added and dropped into the oil-phase serially (1 mL). Beads were left at oil-phase for 2.5 min for spherical shaping to be completed, washed with 0.01% Triton-X and put into the aqua-phase (ultra-pure water) and left for 5 min. Finally, beads were mixed with whey medium (5 mL).

κ-carrageenan: 1.5 g κ-carrageenan was mixed with 75 mL ultra-pure H_2O and autoclaved at 121°C for 15 min. Polymer was cooled down to 50-55°C and then 100 µL of bacteria (1 x 10^7 cfu/mL) was added and dropped into the oil-phase serially (1 mL). Beads were left at oil-phase for 2.5 min for spherical shaping to be completed, washed with 0.01 Triton-X and put into the aqua-phase (0.3 M KCl) and left for 5 min. Finally beads were mixed with whey medium (5 mL).

The protocol of Nillson et al. was used for immobilization in polyacrylamide and Sephadex [15]. Polymerization of acrylamide was not possible. Increasing the concentrations of ammonium persulfate and tetramethylenediamine which are both toxic to cells would not be appropriate, therefore this method was abandoned. Polymerization of Sephadex was achieved but beads were disintegrated. This method was also not used.

***Fermentation*:** Fermentation medium for non-immobilized floating cells was obtained as follows. Five mL of WP-F was mixed with 100 µL bacteria (1x10^7 cfu/mL). Immobilized and free cells were left for fermentation at 40-42°C in an incubator for 24 and 48 hours. After completion of the fermentation, fermentation media of free cells were centrifuged at 20,000 x g and 4°C for 15 minutes. The supernatants were collected and cells were stored in 0.9% saline at – 20°C. Beads of κ-carrageenan, agar and agarose were centrifuged at 500 x g and 4°C for 5 minutes to separate cells from the immobilization medium. Supernatants were collected and centrifuged at 20,000 x g and 4°C for 15 minutes. Cells were stored at – 20°C in 0.9% saline. All experiments were conducted in duplicate.

McFarland standard (0.5, 1x10^8 cfu/mL) was used for spectrophotometric enumeration of bacteria and a cell concentration of 1 x 10^7 cfu/mL was used for fermentation media [16]. Total protein concentration of supernatants after fermentation was measured using Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis {SDS-PAGE] analysis of whey proteins was performed using 12% gel and Coomassie Brilliant Blue staining.

The study was approved by Marmara University Ethics Committee (Approval no. 09.2022.245).

Statistical Analysis

Statistical analysis of data was performed with Prism 7.04 (GraphPad Software Inc., USA).

3. RESULTS

Table I shows total protein concentrations after fermentation of whey proteins with *S. thermophilus* in different media. Total protein concentration of κ-carrageenan immobilization was decreased compared to other media at the end of 24 hours of fermentation. No significant change was observed between two different fermentation periods (24 and 48 hours). We have performed SDS-PAGE analysis to observe changes in individual whey proteins after fermentation in different media (Figure 1). Both α-lactalbumin and β-lactoglobulin were decreased at the end of 24 hours of fermentation in all media. The greatest reduction was in κ-carrageenan compared to other media and control. There was no significant difference in color intensity at the end of 48 hours compared to 24 hours.

Table I. *Total protein concentrations 24 hours after fermentation of whey proteins with S. thermophilus in different immobilization media*

Immobilization media	Total protein (mg/mL)
None	2.035 ± 0.078
Agar	1.115 ± 0.097
Agarose	1.180 ± 0.050
κ-carrageenan	0.771 ± 0.031

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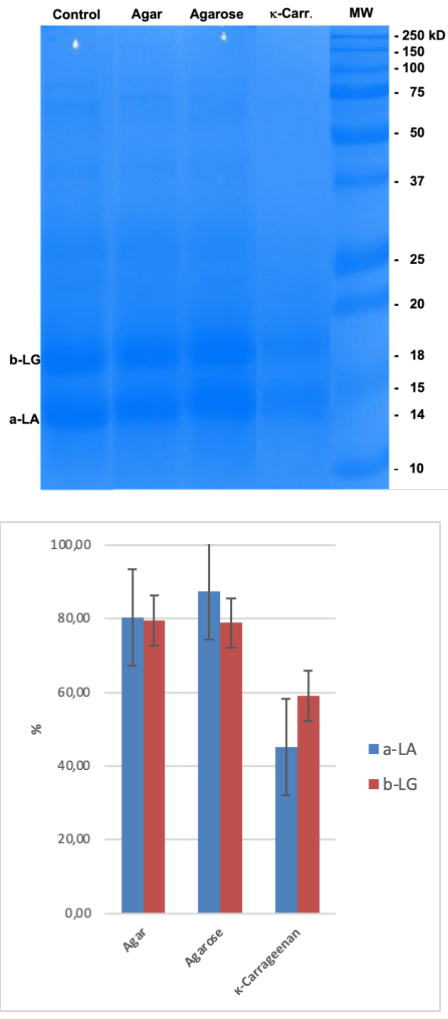

Figure 1. *SDS-PAGE analysis of whey proteins after fermentation with S. thermophilus.*
Lane 1: Control; Lane 2: Agar; Lane 3: Agarose; Lane 4: κ-Carrageenan; Lane 5: MW Markers

The graph shows the results of densitometric scanning and percentage decrease in the intensity of α-lactalbumin and β-lactoglobulin bands compared to control.

4. DISCUSSION

Food allergy is an important health problem and includes the adverse health effects in which immunological mechanisms are involved [17]. Most cases of food allergy are associated with a limited range of products. The most commonly allergenic foods are considered to be cows' milk, hens' eggs, peanuts, tree nuts, soy, wheat, shellfish and fish [18]. Fermentation of milk and milk allergens (such as α-lactalbumin, β-lactoglobulin, α-casein and β-casein), with Lactobacilli strongly reduces their allergenicity [19].

We have observed that the amount of both α-lactalbumin and β-lactoglobulin were reduced in all immobilization media used for fermentation with *S. thermophilus*. The effect was considerably higher on κ-carrageenan compared to the two other media. Immobilization refers to the prevention of free cell movement by natural or artificial means. It has been assumed that cells are distributed homogeneously in the beads that entrap them. However, in a study by Zohar-Perez et al., distribution of *E. coli* in alginate-gel beads was found to be nonhomogeneous [20]. In fact, there was a greater presence of cells on the surface of the alginate beads than in their cores. Similar effects may be responsible for the difference we have observed in different media.

Garbayo et al., hypothesize that immobilization alters cell wall-membrane due to a kind of gel-matrix-structure recognition increasing the permeability and lactic acid production [21]. Thus, use of immobilization medium seems to have potentially high value for whey processing on the commercial scale. Takata et al., reported results confirming the superiority of κ-carrageenan over other immobilization media [8]. In this study κ-carrageenan was far more efficient than agar and agarose in the destruction of β-lactoglobulin found in whey protein by *S. thermophilus*. The use of immobilization medium has the potential to reduce the allergenicity of fermented milk products. Therefore, in biotechnological processes based on the fermentation of milk and dairy products, κ-carrageenan can replace immobilization media such as agar and agarose, which have lower efficiency in reducing the components that pose a risk to health.

Compliance with Ethical Standards

Ethical Approval: The study was approved by Marmara University Ethics Committee (Approval no. 09.2022.245).

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Conflict of Interest : The authors have no conflict of interest to declare.

Author contributions: FZS, GB, and ASY: Generated the initial idea and experimental design, FZS, GB., AMY, and BA: Performed the experiments and analyzed data¸ FZS, GB, and ASY: Wrote the manuscript. All authors contributed to the critical revision and gave final approval to the submitted version.

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