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# Exopolysaccharide Production and Prevention of Syneresis in Starch Using Encapsulated Probiotic *Lactobacillus plantarum*

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**Summary**

Encapsulation of probiotic bacteria with a matrix can increase their survival rate by protecting them from adverse conditions and at the same time without affecting the production of metabolites. An effort has been made to encapsulate the probiotic *Lactobacillus plantarum* using calcium alginate. Box-Behnken model of response surface methodology (RSM) was employed in the optimization of major encapsulation conditions such as concentration of sodium alginate, calcium chloride and curing time. The second-order quadratic model with the optimum conditions (sodium alginate 2 % (by mass per volume), calcium chloride 0.5 M and curing time 3 h) resulted in a maximum titre of (0.9±0.1) g/L of exopolysaccharides (EPS) at 72 h. The nearness of the coefficient of determination ($R^2$=0.97) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The efficiency of EPS production by encapsulated cells was compared with free cells. The efficacy of secreted EPS in the prevention of syneresis in starch was investigated.

*Key words:* encapsulation, exopolysaccharide, lactic acid bacteria, probiotics, response surface methodology (RSM), syneresis

## Introduction

Exopolysaccharides (EPS) from lactic acid bacteria contribute to specific rheology and texture of fermented milk products and find applications even in non-dairy foods and in therapeutics (*1*,*2*). Therefore, EPS-producing strains are of commercial value for both their technological and putative probiotic properties. Microbial polysaccharides of economic interest are usually produced at the industrial level by fermentation. Problems associated with the reproducibility of fermentation during the industrial scale-up may result in inconsistent productivity, yield and quality, all of which can translate into financial losses. This can be solved by optimising the inoculation methodology and the fermentation conditions. A high EPS production could result in the formation of a highly viscous fermentation broth, making the recovery of cells and further downstream processing difficult (*3*). Encapsulation of cells makes easier the biomass separation and it can also be reused.

Encapsulating probiotic bacteria with a matrix that does not interfere in the production of a metabolite can protect the friendly bacteria against the harsh conditions of the stomach and upper intestine, allowing for greater delivery of these value-added ingredients. Increasing research has focused on protecting probiotics during processing and expanding the food categories available to prebiotics. Such an avenue of research has led companies like Cell Biotech from Korea to use a dual coating to protect probiotics against oxygen, acid, moisture and high temperatures and for their use in emerging new product categories such as breakfast cereals and smoothies, which are marketed under the brand name Duolac™.

Starch is one of the most abundant and widely distributed components in foodstuffs. Its gelatinization is commonly achieved by cooking in the presence of water.

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On cooling, starch granules recrystallize to form a solid gel, a process known as retrogradation. A decrease in temperature causes a reduction in the kinetic energy that facilitates the amylose molecules to associate and form a three-dimensional network. As a consequence, water is squeezed out of the gel (syneresis), while intermolecular interaction between amylose molecules becomes stronger and the gel shrinks. Syneresis negatively affects the functional and sensory properties of foods (*4,5*). It should be minimized without interfering with the native properties of food products. This could be achieved with the addition of hydrocolloids such as exopolysaccharides.

An attempt has been made to encapsulate the probiotic strain isolated from curd with calcium alginate, the most commonly used matrix for immobilizing enzymes and cultures, to investigate the effect of encapsulation on the production and release of EPS. The efficacy of secreted EPS in preventing syneresis is also investigated.

## Materials and Methods

### *Bacterial strains and media*

*Lactobacillus plantarum* MTCC 9510 is a facultative anaerobe isolated from curd and was sub-cultured in de Man–Rogosa–Sharpe (MRS) medium (Himedia, Mumbai, India) at 37 °C for 24 h under static conditions. It was maintained in MRS agar at 4 °C for short term use and glycerol stocks were made for preservation. The culture isolated from the curd was selected for the study because it exhibited most of the probiotic features (*6*) with a quantifiable yield of exopolysaccharides. The isolated culture was evaluated for a number of probiotic characteristics like bile salt resistance, salt tolerance, survival in low pH, hydrophobicity of the cell surface, resistance to low phenol concentration, antimicrobial activity and susceptibility pattern against vancomycin and erythromycin. *L. plantarum* had 23 % hydrophobicity, proving the ability of cells to adhere to hexadecane droplets. It tolerated 12 % salt, 0.5 % phenol and 0.8 % bile salt and survived even at a low pH of 2.5. It also showed antimicrobial activity against potent human gastrointestinal pathogens like *Shigella sonnei*, *Shigella flexneri*, *Staphylococcus aureus* and *Escherichia coli*. Antibiotic resistance studies showed that the isolate was resistant against vancomycin and sensitive to erythromycin. It also showed an emulsification index of 33 % with emulsion stability at hot and cold temperatures and even at higher concentration of sodium chloride.

### *Encapsulation of Lactobacillus plantarum and fermentation conditions*

The culture ($10^9$ CFU/mL) was inoculated into MRS broth and incubated at 37 °C for 18 h to prepare the inoculum. A volume of 25 mL of inoculum was centrifuged at 8000 rpm for 10 min at 4 °C. The cells were then washed with physiological saline. The pellet was resuspended in 5 mL of sterile distilled water and mixed with 5 mL of sterile sodium alginate (1, 2 and 3 %, by mass per volume). The mixture was dropped into 25 mL of calcium chloride (0.1, 0.5 and 1 M) using a syringe (needle size 0.70×32 mm) and left for curing (2, 3 and 4 h) at 4 °C depending on the experimental set-up. The fermentation was conducted in 250-mL Erlenmeyer flask using 50-mL modified EPS production medium containing (in g/100 mL): yeast extract 4.0, lactose 4.0, Tween 80 0.1, sodium acetate 0.5 and ammonium sulphate 0.5. The inoculated flasks were incubated at 37 °C for 72 h under static conditions. Cell leakage was checked at regular intervals by measuring the absorbance of the fermented broth at 620 nm.

### *Extraction and estimation of EPS*

Exopolysaccharides were extracted according to the method of Aswathy *et al*. (*6*), after boiling the culture at 100 °C for 10 min to inactivate the EPS-degrading enzymes. The EPS produced were calculated as the total carbohydrate content in the extract by phenol sulphuric acid assay (*7*).

### *Box-Behnken design and data analysis*

A fractional factorial design, Box-Behnken model, was employed for the statistical optimization of the encapsulation conditions. The experimental design consisted of seventeen runs and the independent variables were studied at two different levels, a high level and a low level. The high level is commonly coded as +1 and the low level as -1. It is necessary to include centre points as well (in which all factors are at their central values). The response variable (EPS) was fitted by a second order model to correlate the response variable to the independent variables. The general form of the second degree polynomial equation is:

$$Y_i = b_0 + b_i \Sigma X_i + b_{ii} \Sigma X_i^2 + b_{ij} \Sigma_i \Sigma_j X_i X_j \qquad /1/$$

where $Y_i$ is the predicted response, $X_i$ and $X_j$ are input variables which influence the response variable Y, $b_0$ is the offset term, $b_i$ is the $i^{th}$ linear coefficient, $b_{ii}$ is the quadratic coefficient and $b_{ij}$ is the $ij^{th}$ interaction coefficient. Three factors selected for optimization were concentrations of sodium alginate and calcium chloride along with the time for curing. A design was generated with these factors, having a low level and a high level. The low level for sodium alginate was 1 % and the high level was 3 %. The low level for calcium chloride was 0.1 M with a high level of 1 M. The low and high level values for curing time were 2 and 4 h, respectively. The Design Expert software (v. 6.0, Stat-Ease, Inc., Minneapolis, USA) was used for experimental design (Table 1), regression and graphical analyses of the obtained data. The maximum value of the produced EPS was taken as the response.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its associated probability p(*F*), correlation coefficient R, and determination coefficient $R^2$ which measures the goodness of fit of the regression model. It also includes the Student's *t*-value for the estimated coefficients and associated probabilities, p(*t*). The quadratic models were represented as response surface graphs. Validation of the experiment was also performed by selecting different combinations of the factors as recommended by the software. All the experiments were performed in triplicates.

Table 1. Experimental design generated with Design Expert software and the predicted and actual values of production of exopolysaccharides

| Run | *w*(sodium alginate) | *c*(calcium chloride) | Curing time | $\gamma$(actual EPS) | $\gamma$(predicted EPS) |
|---|---|---|---|---|---|
| | % | M | h | g/L | g/L |
| 1 | 3 | 0.55 | 2 | 0.20 | 0.23 |
| 2 | 1 | 1.00 | 3 | 0.10 | 0.21 |
| 3 | 3 | 0.55 | 4 | 0.64 | 0.68 |
| 4 | 3 | 0.10 | 3 | 0.83 | 0.72 |
| 5 | 2 | 0.55 | 3 | 0.94 | 0.94 |
| 6 | 2 | 0.55 | 3 | 0.94 | 0.94 |
| 7 | 2 | 0.55 | 3 | 0.94 | 0.94 |
| 8 | 2 | 1.00 | 2 | 0.23 | 0.16 |
| 9 | 2 | 1.00 | 4 | 0.25 | 0.17 |
| 10 | 2 | 0.10 | 4 | 0.45 | 0.52 |
| 11 | 1 | 0.10 | 3 | 0.34 | 0.30 |
| 12 | 1 | 0.55 | 4 | 0.13 | 0.09 |
| 13 | 2 | 0.55 | 3 | 0.94 | 0.94 |
| 14 | 2 | 0.10 | 2 | 0.14 | 0.22 |
| 15 | 3 | 1.00 | 3 | 0.35 | 0.39 |
| 16 | 2 | 0.55 | 3 | 0.94 | 0.94 |
| 17 | 1 | 0.55 | 2 | 0.26 | 0.22 |

standard deviation was found to be ±5 %

### *Comparison of free and encapsulated Lactobacillus plantarum*

The efficiency of free and encapsulated cells in EPS production was investigated. Two different inoculum ($10^9$ CFU/mL) volumes were used in the experiment, 1 and 25 mL of 18-hour-old inoculum. Fermentation was carried out with free inoculum and also with encapsulated cell beads made from the biomass. The efficiency of free and encapsulated cells of both inoculum volumes was monitored. After inoculation, the flasks containing 50 mL of EPS-production medium were incubated at 37 °C under static conditions, and EPS production by free and encapsulated cells followed at regular intervals up to 72 h. Initially, the experiment was performed to study the efficiency of cells when they remain free or encapsulated at two extreme inoculum strengths (1 and 25 mL) rather than selecting a series of closely related inoculum strengths. A repeated batch study with encapsulated cells from 25 mL of inoculum was also conducted.

### *Prevention of syneresis in starch*

The ability of exopolysaccharides (EPS) produced by *Lactobacillus plantarum* to minimize the liquid separation (syneresis) of cooked starch pastes during refrigeration was investigated. The extent of syneresis was estimated according to the protocol mentioned by Viñarta *et al.* (*8*). The liquid phase length ($\Delta h$) separated above the sedimented phase was measured in two-day intervals throughout the storage at 4 °C for 20 days. The degree of syneresis was represented by $\Delta h/h_0$, where $h_0$ stands for the initial height of the sample dispersion. Two different starch systems were chosen for the study, cassava starch and wheat starch. The starch powder (2 %) was dispersed in distilled water and cooked for 10–15 min in a boiling water bath with gentle mixing until the paste thickened. A blend with polysaccharide dispersion (0.2 %) was made. The efficacy of EPS from *L. plantarum* was compared with a control, carboxymethyl cellulose (CMC, Sigma Aldrich, St. Louis, MO, USA). Polysaccharide dispersion was made separately by dispersing the EPS (0.2 %) in distilled water and then stirred in a magnetic stirrer until complete dissolution. Sodium benzoate or sodium metabisulphite was added to the mixture to prevent microbial activity (1 g/L). The mixture was allowed to reach room temperature before being aliquoted. The starch samples or mixture (starch and polysaccharide) were poured into screw-cap plastic tubes to reach a final volume of 15 mL. Tubes were held vertically at 4 °C for 20 days, unless otherwise stated, and the extent of syneresis was measured in two-day intervals.

## Results and Discussion

### *Box-Behnken design and data analysis*

Three variables (sodium alginate concentration, calcium chloride concentration and curing time) were selected to investigate the optimum combination of factors suitable for encapsulating cells using calcium alginate. The Box-Behnken model of RSM was employed in the experiment. There was a total of seventeen runs based on the model generated by the software, each in triplicate. The experimental data were statistically analysed using the Fisher's statistical test for analysis of variance (ANOVA) and the 3D graphs were designed. The ANOVA explains the model to be significant with an *F*-value of 24.51. There is only a 0.02 % chance that a model *F*--value this large could occur due to noise. Adeq Precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The ratio of 12.183 indicates an adequate signal. The goodness of fit of the model was checked by determination coefficient ($R^2$). In this case, the value of the determination coefficient ($R^2$=0.97) indicates the significance of the model. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case lower value of CV (17.81) indicated a greater reliability of the performed experiments. The p-values denote the significance of the coefficients and are also important in understanding the pattern of the mutual interactions between the variables. Value of Prob>F less than 0.05 indicates that the model terms are significant. Here the terms $X_1$, $X_2$, $X_3$, $X_1^2$, $X_2^2$, $X_3^2$ and $X_1X_3$, *i.e.* the linear effect of sodium alginate, calcium chloride and curing time, quadratic effect of all the factors and the interaction effect of sodium alginate and curing time, are significant.

The polynomial equation derived on the basis of the experimental factors, quadratic effect of the factors and the interactions among the factors by the input of values in Eq. 1 is shown below:

$$Y=0.94+0.15X_1-0.10X_2+0.080X_3 -0.25X_1^2-0.29X_2^2-0.39X_3^2 -0.060X_1X_2+0.14X_1X_3-0.073X_2X_3 \quad /2/$$

where Y is the response, *i.e.* exopolysaccharides, and $X_1$, $X_2$ and $X_3$ are the coded values of the test variables sodium alginate, calcium chloride and curing time, respectively. The probability values of 0.0023, 0.014 and 0.04 for sodium alginate, calcium chloride and curing time, respectively, ensure the factors to be significant in the encapsulation of cells and in the release of EPS. The quadratic effect of all the factors and the interaction effect of sodium alginate and curing time was found to be significant, indicating that the interaction between sodium alginate and curing time is necessary for the encapsulation conditions and in the final release of EPS.

The RSM is shown as 3D response surface graphs, which gives infinite number of combinations of the two factors selected, keeping the third factor constant. The maximum amount of EPS ((0.9±0.1) g/L) was released by the encapsulated cells in a combination of sodium alginate 2 %, calcium chloride 0.5 M and curing time 3 h with minimum cell leakage. It was noted that the amount of released EPS was higher with this combination and also the cell leakage at regular intervals was lower compared to the rest of the compilations. The interaction effects between sodium alginate and calcium chloride, and between calcium chloride and curing time were not found to be significant (data not shown). However, the ANOVA showed that the interaction effect of sodium alginate and curing time (Fig. 1) was significant in the encapsulation of cells and in the release of EPS through the matrix. The appropriate concentration of sodium alginate was found to be 2 %. The higher the concentration of alginate, the lower the death rate of cells in beads, as well as the lower diffusion rate of sugar into the beads. Therefore, the concentration of alginate should be medium, supporting the obtained result. The lower diffusion rate of glucose in more concentrated alginate gels is due to a decrease in the number and length of pores rather than to a decrease in the pore diameter (*9*). In the experiment, curing time of 3 h was found to be optimum for the encapsulation of cells and EPS release. Increasing curing time will result in harder beads with less EPS release, hence the curing time should not be lower or higher.

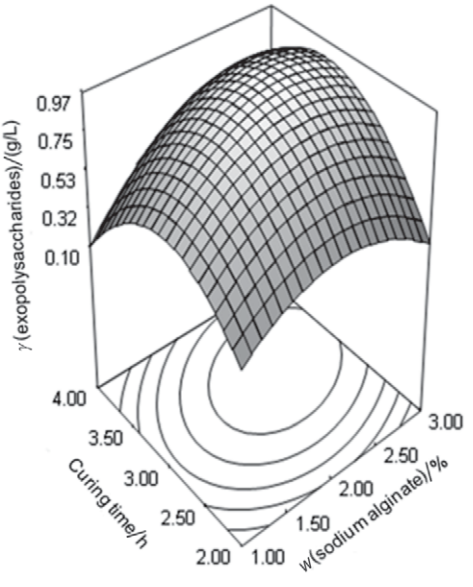


**Fig. 1.** Interaction effect of sodium alginate and curing time in the release of EPS produced by encapsulated *Lactobacillus plantarum*

### *Validation of the model*

The model was validated in shake flask experiment using the conditions predicted by the software. The results showed actual values nearer to the predicted values, supporting the data and the model as valid. The maximal production observed during validation was 0.9 g/L as in the initial experiment against the predicted value of 0.94 g/L.

A few of the known LAB that are used as probiotics are *Lactobacillus acidophilus*, *Lactobacillus amylovorous*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *etc*. (*10*). The organism used in the present study for encapsulation is *L. plantarum*. Based on the organism and the product of interest, the alginate concentration required for immobilization may differ (*11–13*), and hence it has to be optimized accordingly. Concentration and type of alginate used for immobilization determine the properties of beads, as alginate may contain different proportions of mannuronic and guluronic acids (*14*). It was found in the experiments that the appropriate amount of alginate required for the proper encapsulation of *L. plantarum* cells was 2 %. Alginate forms a gel in the presence of divalent cations, such as $Ca^{2+}$, which link specifically to the G-blocks (guluronic acid blocks) by binding to the free carboxyl groups. The mechanical stability of the alginate bead is critical in the physiologic environment where divalent ions, which are involved in the network formation, are exchanged with other ions in the environment. This results in a loosening of the G-G bonds, with a subsequent increase in porosity, swelling of the capsule, and release of the biomaterial or cell (*15*). Alginate beads are unstable in the presence of other metal ions like $Mg^{2+}$ or $K^+$ (*5,8*). Hence, attention was taken to modify the production medium in such a way that it is devoid of potassium and magnesium ions. Optimum conditions of the selected variables were defined on the basis of the maximum amount of EPS released and with minimum cell leakage. The behaviour of entrapped cells depends on the nature of biological material in beads (*16*).

In a broad sense, encapsulation can be used for many applications in the food industry, including stabilizing the core material, controlling the oxidative reaction, providing sustained or controlled release (both temporal and time-controlled release), masking flavours, colours or odours, extending the shelf life and protecting components against nutritional loss. It is clear from the experiment that the used matrix, calcium alginate, was proper for the encapsulation of *L. plantarum* as there was minimum leakage of cells and maximum release of EPS without interfering with its production and release.

### *Comparison of free and encapsulated Lactobacillus plantarum*

A comparison of free and encapsulated cells was done to see the influence of both types of cells on the EPS production. On comparison it was found that the encapsulated inoculum volume of 25 mL gave threefold EPS production than the 1 mL. The reason could be larger amount of viable inoculum. One of the advantages of encapsulation technology is the possibility of supplying fermentation reactions with larger viable inoculum (biomass). It was clear from the experiment with inocula of 25 and 1 mL that in both cases the encapsulated cells gave higher production (Fig. 2) than the free cells of the same inoculum volumes in batch fermentation. This could be because the competition between cells is smaller when encapsulated than in the case of free cells. It was concluded from the study that higher amount of encapsulated inoculum can increase the production. A repeated batch study with the encapsulated *L. plantarum* showed similar production even in the second batch, confirming the viability and efficiency of encapsulated cells.

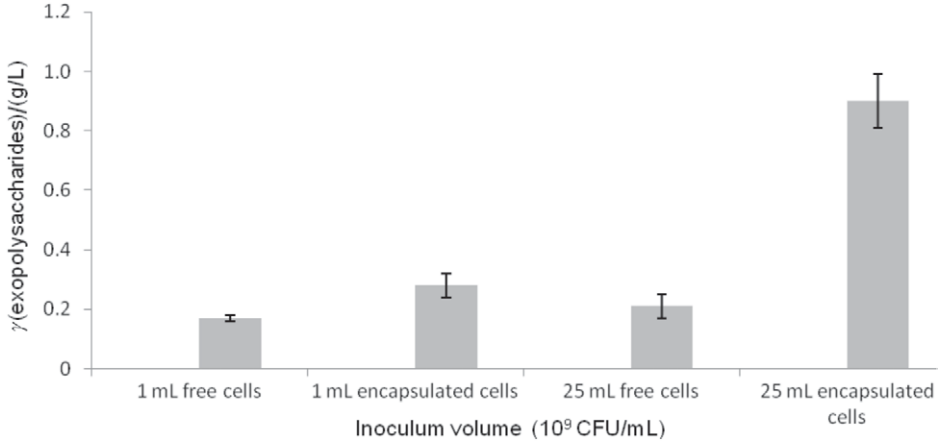


**Fig. 2.** Comparison of free and encapsulated *Lactobacillus plantarum* and inoculum volume in the efficiency of EPS production

### *Prevention of syneresis in starch*

Out of the two different starches used here, only wheat starch was found to have some sensitivity towards syneresis. Cassava starch is a tuber starch while wheat starch is a grain starch. Cassava starch is oval truncate in shape with an average size of 25 μm while the other is round lenticular of the same size. The tuber and root starches have lower fat content than the grain starch. It has already been reported that the cassava starch exhibits high stability to cold storage (*5*). Hence, wheat starch was chosen for the rest of the studies. The EPS from *L. plantarum* was found to have better influence on the prevention of syneresis in wheat starch when compared with the control polysaccharide, carboxymethyl cellulose (Fig. 3). The concentration of starch and EPS in the experiment was 2 and 0.2 %, respectively. The EPS prevented syneresis in a concentration of 0.2 % (by mass per volume). Increasing the concentration of EPS can have a profound influence on the prevention of syneresis. This has to be further optimized. Positive effects may be attained at levels as low as 0.1–1 % (*4*,*17*,*18*). The open structure produced by some EPS-producing cultures (*19*,*20*) might induce syneresis. However, EPS have the ability to bind water (*21*), which counteracts the negative effect of the open structure. EPS prevent syneresis and improve product stability firstly by increasing the viscosity and elasticity of the final product, and secondly by binding hydration water. It is speculated that the increased viscosity of EPS-containing foods may increase the residence time of ingested fermented milk in the gastrointestinal tract and therefore be beneficial to a transient colonisation by probiotic bacteria. Several methods, with and without centrifugation, are available for the evaluation of syneresis. Since the method involving centrifugation has already been criticized (*4*), a method without centrifugation was considered for the experiment. This methodology showed to be a reliable, highly accessible, inexpensive and labour-saving, as no sophisticated instruments are required for the evaluation.

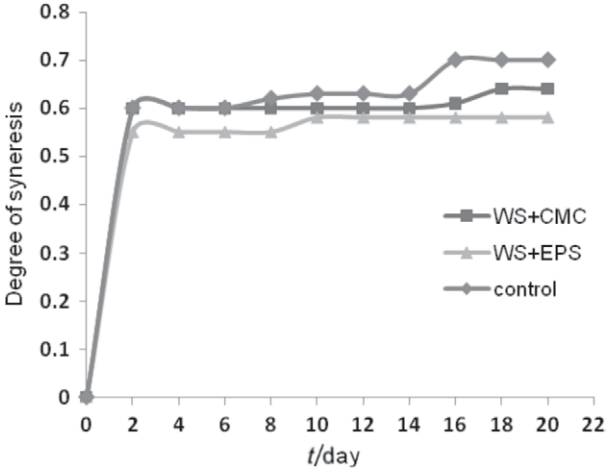


**Fig. 3.** Syneresis *vs.* refrigeration at 4 °C for wheat starch. WS – wheat starch, control – wheat starch and water, CMC – carboxymethyl cellulose, EPS – exopolysaccharides from *Lactobacillus plantarum*

## Conclusion

In conclusion, the encapsulation technology was found to be an effective method for retaining the efficiency of cells even in repeated batches. The EPS had profound influence on the prevention of syneresis in wheat starch, imparting that either the EPS-producing encapsulated probiotic strain *Lactobacillus plantarum* or the extracted EPS can act as an ingredient in the products that can be used for consumption.

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