Research Article

APPLIED FOOD BIOTECHNOLOGY, 2020, 7(3): 171-182
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Incorporation of *Lactobacillus casei* into the Inner Phase of the Water-in-Oil-in-Water ($W_1/O/W_2$) Emulsion Prepared with β-Cyclodextrin and Bacterial Survival in a Model Gastric Environment

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Abstract

Background and objective: Literature contains extensive researches on use of multiple emulsions in a variety of areas such as foods, pharmaceuticals, and health products. Water-in-oil-in water double emulsion ($W_1/O/W_2$) was used in the present study for entrapping *Lactobacillus casei* where balance in gastric system is under influence of probiotics release mechanism. Role of β-cyclodextrin emulsifier presented in the external aqueous phase of the test emulsion was explainable through emulsion characterization analyses (Fourier Transform Infrared spectroscopy, particles size distribution and Scanning Electron Microscopy). Cells viability was determined in terms of first-order kinetics and by applying the probability concept and survivability in gastric system was evaluated probabilistically using the exponential distribution.

Material and methods: Three different ratios of $W_1/O:W_2$ [40:60, 50:50, 60: 40] were used for preparing $W_1/O/W_2$ emulsions to encapsulate *Lactobacillus casei* into the inner phase with using β-cyclodextrin emulsifier. Dynamic light scattering was used for determination of emulsion particle size. Presence of the functional groups was detected using infrared spectroscopy and recording the relevant peaks in the region of 4000 to 400 cm^{-1}. The differential equation for first-order kinetic ($dx/dt = kx$) was used for the cells viability determination. Exponential probability distribution in terms of failure time (reflection of the cells not being able to be released) was also used in this study ($f(t) = \lambda e^{-\lambda t}$).

Results and conclusion: By preparation of gastric environment in terms of the bile salts and in a pH-controlled media, viability of the encapsulated cells was monitored and the following expression for the first-order rate constant was obtained for the test emulsions ($k_{(60:40)} < k_{(50:50)} < k_{(40:60)}$). Emulsification appears to be feasible industrially since the composition of the emulsions used for *Lactobacillus casei* entrapment was entirely edible.

Conflict of interest: The authors declare no conflict of interest.

Article Information

Article history:
Received 01 Feb 2020
Revised 19 May 2020
Accepted 31 May 2020

Keywords:
- Exponential distribution
- Gastric environment
- Kinetic modeling
- *Lactobacillus casei* viability
- Water-in-Oil-in-Water ($W_1/O/W_2$) double emulsion

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How to cite this article

Eslami P, Forootan K, Davarpanh L, Vahabzadeh F. Incorporation of Lactobacillus casei into the Inner Phase of the Water-in-Oil-in-Water ($W_1/O/W_2$) Emulsion Prepared with β-Cyclodextrin and Bacterial Survival in a Model Gastric Environment . Appl Food Biotechnol 2020; 7(3): 171-182. http://dx.doi.org/10.22037/afb.v7i3.28877

1. Introduction

Probiotic bacteria have all been recognized (and differentiated from other bacteria) for their capabilities to treat diseases and health problems [1,2]. Special efforts are directed towards finding delivery systems that can efficiently encapsulate bacteria, protect and transport them to human body without cellular damages/side effects [3-7]. Other bioactive compounds as sensitive molecules with oil and water solubility characteristics can be kept in an emulsion system where emulsification technique is a favorable process being used to incorporate these types of

molecules (encapsulation process). The system also can be designed to release the encapsulated core material at a controllable rate to a particular environment (such as release in intestinal tract during food consumption). The designed double emulsion is a part of preservation method able to maintain the compound of the interest away from damaging environment [6]. In fact, double emulsions are not only used in food systems but, they are also applied in a variety of areas such as pharmaceuticals and health products [5]. In Fig. 1, the function of probiotic bacteria in relation to bile acids and bile salts is presented.

The fundamental basis in formation of emulsion system is to follow fate of the hydrophobic interaction between two immiscible liquids, which is considered to be a thermo-dynamically unfavorable process ($\Delta G=\Delta H -T\Delta S=$ (+) value). Supplying energy to this system in the form of $\Delta G=\gamma\Delta A$ shows the importance of increasing contact area (ΔA) between the two solvents and interfacial tension (γ) [8]. Formation of particles through addition of emulsifier to the test system can be interpreted in terms of structural organization (system's entropy, ΔS = (+) value) thus, ΔG becomes negative and emulsification can be categorized as thermodynamically favorable process. Emul-sion droplet properties therefore define the emulsion stabilization phenomenon [8].

Cyclodextrins as the cyclic oligosaccharides are produced by the fermentative action of certain bacteria such as *Bacillus* sp. DSM 2523 possessing cycloglucano transferase enzyme activity [9]. Emulsifying ability of the Cyclodextrins is considered to be related to the formation of surface-active inclusion complexes at the interface of the oil/water [10]. Inclusion complexes can actually be considered as an extension of the emulsifying ability of the nano-sized metal solid particles introduced by Pickering more than fifty years ago.

Emulsification technique has been placed among practicable technologies for probiotics encapsulation, but few studies have focused on use of Pickering type double emulsions for this purpose. While, presence of β-cyclodextrin (β-CD) emulsifier directs aforementioned emulsions towards water-in-oil-in-water ($W_1/O/W_2$) emulsions. In fact, behavior of these types of emulsions with regard to *Lactobacillus* (*L.) delbrueckii* was evaluated in our previous work [5]. Since double emulsions are viscoelastic compounds, their performance in terms of the rheological characteristics in addition to structural properties were determined (using the established instrumental analyses: Fourier Transform Infrared spectroscopy, particle size distribution and scanning electron microscopy and the results thoroughly discussed in that work [5].

Thus, it appeared to us to continue this novel approach towards in-vitro study where, simulating gastric fluid and release of the encapsulated cells were the point of interest. The work was extended by applying probability concept (exponential distribution) to follow the release behavior of the entrapped *L. casei* in that test systems.

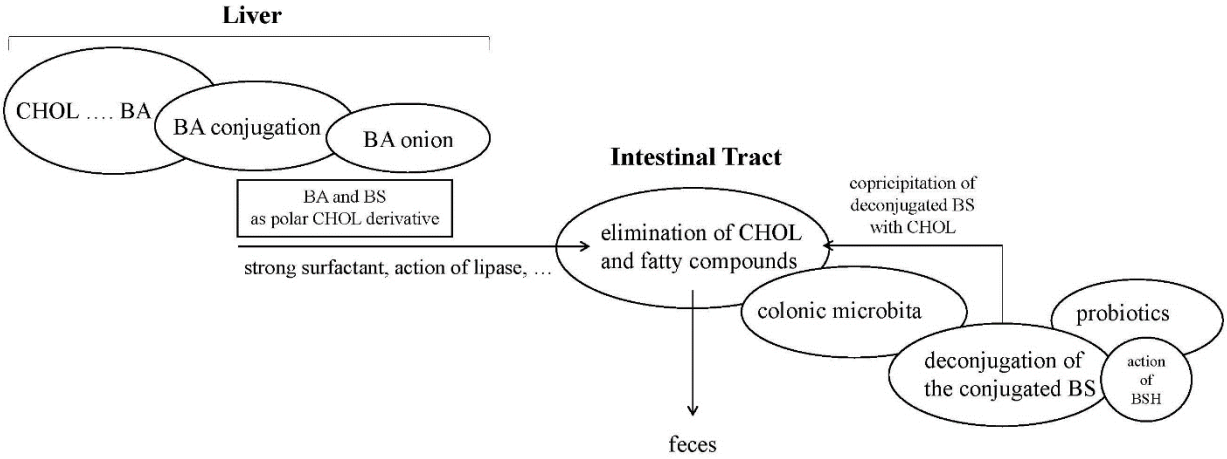


Figure 1. Probiotic behavior in relation to bile acids, bile salts, binding to amino acids (conjugation), deconjugation, intestine and cholesterol removal. No attempts were made to show liver relations with blood circulatory system, tissues, and enter hepatic circulation

2. Materials and methods

2.1. Materials

L. casei ssp. *casei* (PTCC 1608) as the Lactic acid bacteria (LAB) was purchased in the lyophilized form from Iranian Research Organization for Science and Technology (IROST). β-CD, Span 80 and the mixture of sodium cholate and sodium deoxycholate as the bile salts, all were analytical grade from Merck and Sigma-Aldrich. Other chemicals including corn oil were obtained from the local markets.

2.2. Cultivation of *L. casei* and inoculum preparation

Lyophilized culture of *L. casei* was rehydrated in MRS broth and incubated anaerobically in an anaerobic jar (BD BBL™ Gas Pak™ Jar) at 37°C for 48 h (stock culture). Growth curve for *L. casei* was determined spectrophotometrically by withdrawing an appropriate aliquot of the grown culture from the stock solution at the regular time interval and reading its optical density at 600 nm.

Further note was to obtain values of colony forming unit in terms of CFU ml^{-1}. An appropriate volume from the stock culture was withdrawn and dispersed over MRS agar plate (0.1 ml) and this was followed by the incubation of the prepared plates anaerobically at 37°C for 24 h. The colony counting procedure as the standard method was practiced and the determined value for CFU was about 1×10^{11}. The value so obtained was the basis for the amount of the cells used for the preparation of the $W_1/O/W_2$ emulsions.

The cells from the stock culture were harvested using centrifugation (1098 ×g for 10 min) and the sediment was washed using sterile PBS solution (phosphate buffer saline). The centrifugation process was repeated three times and the cell suspension (presumably containing 1×10^{11} CFU ml^{-1}) was used as the W_1 phase [5].

2.3. $W_1/O/W_2$ emulsions preparation and *L. casei* incorporation

The W_1/O as the primary emulsion consisted of sterile distilled water and corn oil mixture where the *L. casei* inoculum prepared as described in above section, was added to the aqueous phase at the ratio of W_1 to O 50:50. Emulsion formation was carried out using homogenizer (HO4 Edmund Buhler 7400 Tubingen-Germany) at 3226 ×g for 4 min at room temperature. Span-80 at 10 v v^{-1}% was added to the continuous oily phase. Thereafter, double emulsions were prepared at three ratios of W_1/O to W_2 60:40, 50:50, and 40:60. Emulsion formation was complete by adding β-CD emulsifier at 2.5 wv^{-1}% to the W_2 phase. Purity of the encapsulated *L. casei* was periodically checked using Gram-staining procedure as described in the relevant literature.

Structural properties of the formed double emulsions were characterized using instrumental analyses: Fourier Transform Infrared spectroscopy, particle size distribution and Scanning Electron Microscopy [5].

2.4. Particle size determination

The particle size distribution analysis of the $W_1/O/W_2$ emulsions were performed using dynamic light scattering technique by a Mastersizer 2000 (Malvern Instruments, UK) at room temperature.

2.5. Fourier Transform Infrared spectroscopy-Attenuated Total Reflection spectroscopy

Confirmation of the functional groups presence in emulsion samples as well as IC particles formed by complexation between β-CD and W_1/O phase was performed by Fourier Transform Infrared spectroscopy technique using Thermo Scientific Nicolet iS10 spectrometer (U.S.) with an attenuated total reflection accessory. Spectra were obtained in transmittance mode from 4000 to 400 cm^{-1}.

2.6. Scanning electron microscopy analysis

The structure of the $W_1/O/W_2$ double emulsions was observed by environmental scanning electron microscopy (Philips XL30, Netherlands).

2.7. *L. casei* response to bile salts, acid, and elevated temperature (viability of the entrapped *L. casei*)

The entrapped *L. casei* at CFU equal to 1×10^{11} was added to 10 ml sterile 0.3% bile salts solution and the tubes were incubated in an anaerobic jar at 37°C. Procedure of the sampling lasted for 3 h where every 30 min the samples were withdrawn and checked for the viability test as follows: centrifugation (2240 ×g, 10 min), collection of the sediment, and washing it by phosphate buffer (pH=7). Thereafter, the enumeration procedure for obtaining CFU values was followed. Viability of the entrapped *L. casei* in response to acidic solution (pH=2) was measured by applying the relevant CFU determination procedure. Also, the effect of elevated temperature on the viability of *L. casei* was measured by heating the bacterial solution being entrapped within double emulsion or not being entrapped (free form of the bacteria in the test solution), at 65°C for a certain time period in water bath (15 and 30 min). The test solutions were let to stand at room temperature and thereafter, the viability was determined using the described method as mentioned above.

2.8. Kinetic study and use of exponential probability distribution

First order reaction kinetic was used to describe release of the encapsulated *L. casei:*

Release reaction rate (v) = $-dx/dt = kx$

Where x is the colony forming unit per volume (CFU ml^{-1}) and k is the proportionality constant ($time^{-1}$). The following expression was used for defining k constant:

$k = -dx/x/dt$

The value so obtained would bear useful information about the release of the entrapped bacterial cells.

Exponential probability distribution was also used in the present study according to the following equation:

$F(t) = \lambda e^{-\lambda t}$

where λ scales t (time) reflecting a mean of the random variable (occurrence of the release of the entrapped *L. casei* "CFU ml^{-1}") in the defined time interval (for instance 30 min in the present study) [11].

2.9. Statistical analysis

Tests for each sample were repeated at least two times (preparation of double emulsions and bacterial loading, microbial growth curve and viability tests). The mean value in each case with the standard deviation (±0.05) was reported (Excel software).

3. Results and discussion

3.1. Double emulsion characterization

Different ratios of $W_1/O/W_2$ were used for preparing $W_1/O/W_2$ emulsions. Primary emulsion in the double emulsions behaves as the dispersed phase. Inclusion complexes formed as the result of β-CD presence in the aqueous W_2 phase in the present study ultimately acted as the nano-sized solid particles covering emulsion droplets [12]. Relationship between inclusion complexes and these Pickering types of emulsions has well been described in the literature [13]. Inclusion complexes not being adsorbed and remaining as free form of colloidal particles, may interact with each other and promote emulsion instability explained under depletion flocculation mechanism in the literature [8]. The findings reported by Fernandez-Martin, Freire [14] showed that additional commercial type of mono- and di-glyceride mixture to the $W_1/O/W_2$ for the induction of fat crystallization in the emulsion oil phase did not provide the emulsion stability where the surfactant was insufficient for covering widened surface area. The researchers in that study discussed the results in terms of relationship between $D_{3,2}$ and emulsion stability. Regarding $D_{4,3}$, results of the study on $W_1/O/W_2$ emulsion prepared with mineral oil (emulsifier PGPR90) showed that the emulsion had larger droplet size in terms of $D_{4,3}$ compared to the size values obtained by butter oil and linseed oil, where the release of azocasein peptides was of interest [6]. In a study on presence of texturized whey protein concentrate in aqueous phase of the O/W emulsion prepared with butter oil, the microstructure of the butter oil increased the chance of coalescence when the storage temperature of the emulsion decreased from 25 to 5°C, but the change of $D_{4,3}$ was not observed in the case of corn oil as the dispersed phase [15]. Structure and organization of the involved molecules in the emulsion formation ultimately determined the dynamic nature of the interfaces separating phases in primary emulsion and also in W_1/O from W_2 in double emulsion. In the present study, Fig. 2 shows the size distribution pattern as the result of the inclusion complexes covering the dispersed phase where different sizes of droplets are present in double emulsions ranging from 0.448 to 56.368 μm. That figure also demonstrates two different ways of showing for particle size distribution: a monomodal peak for each of the three $W_1/O:W_2$ ratios used in the emulsion preparation and relevant cumulative plot as the S-shaped curves. In Fig. 2a, values of the median droplet size showing that 50% of the droplets stays above this level and the other 50% is below that level, are presented and size distribution pattern appears to be normal and the span value is usable term for determining size distribution width.

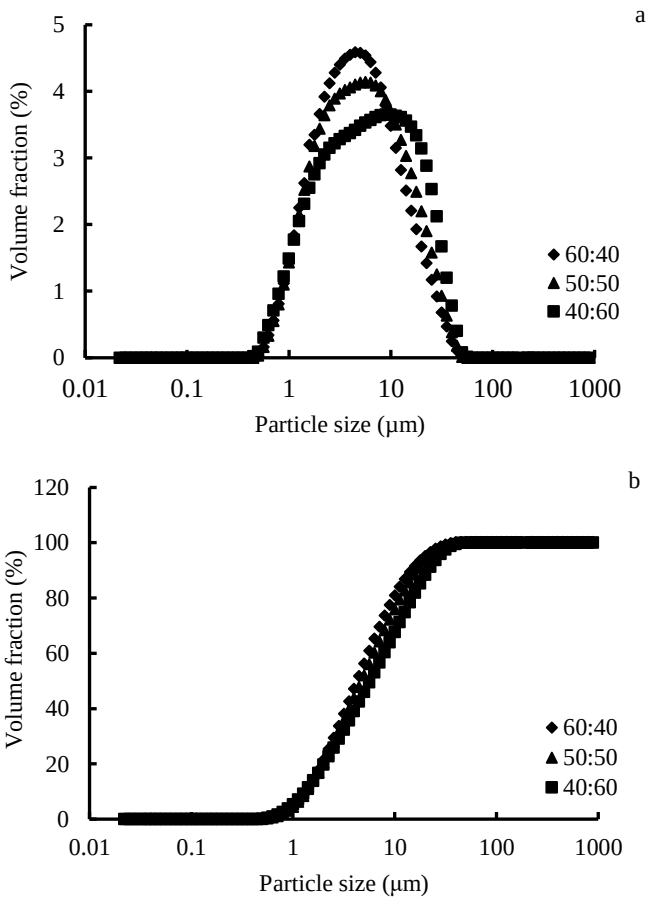
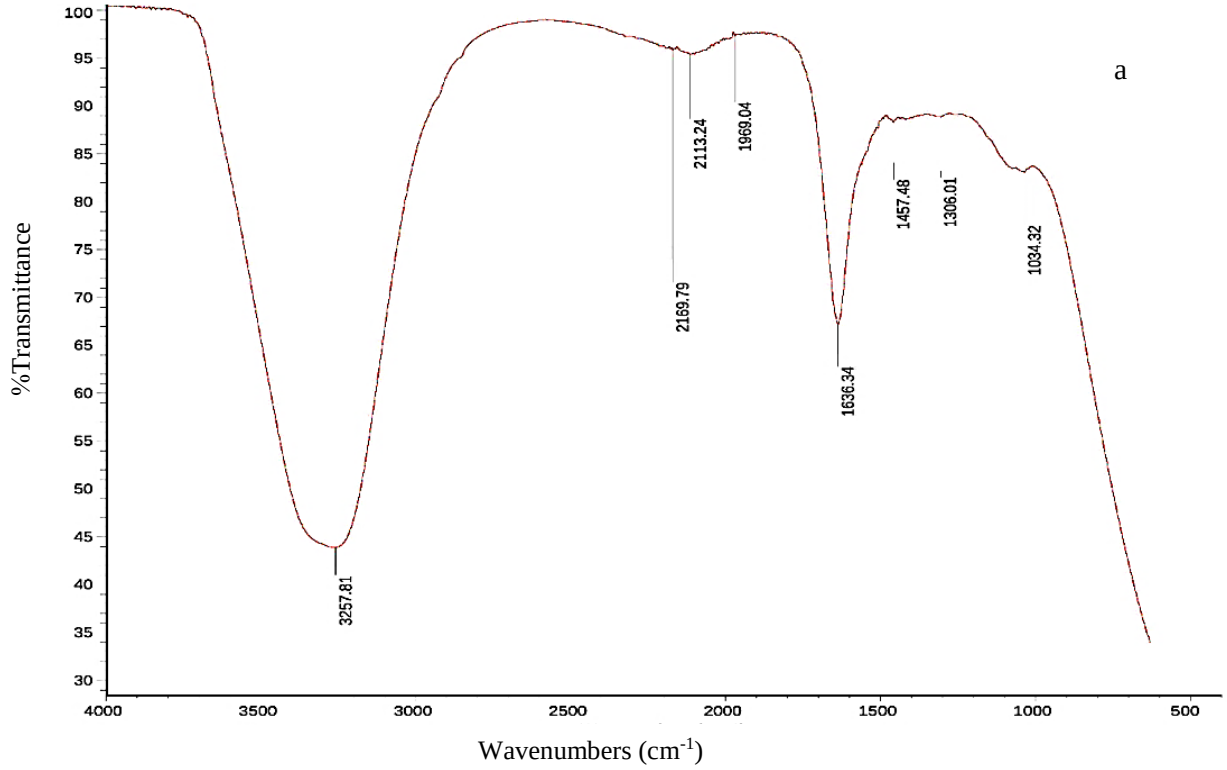
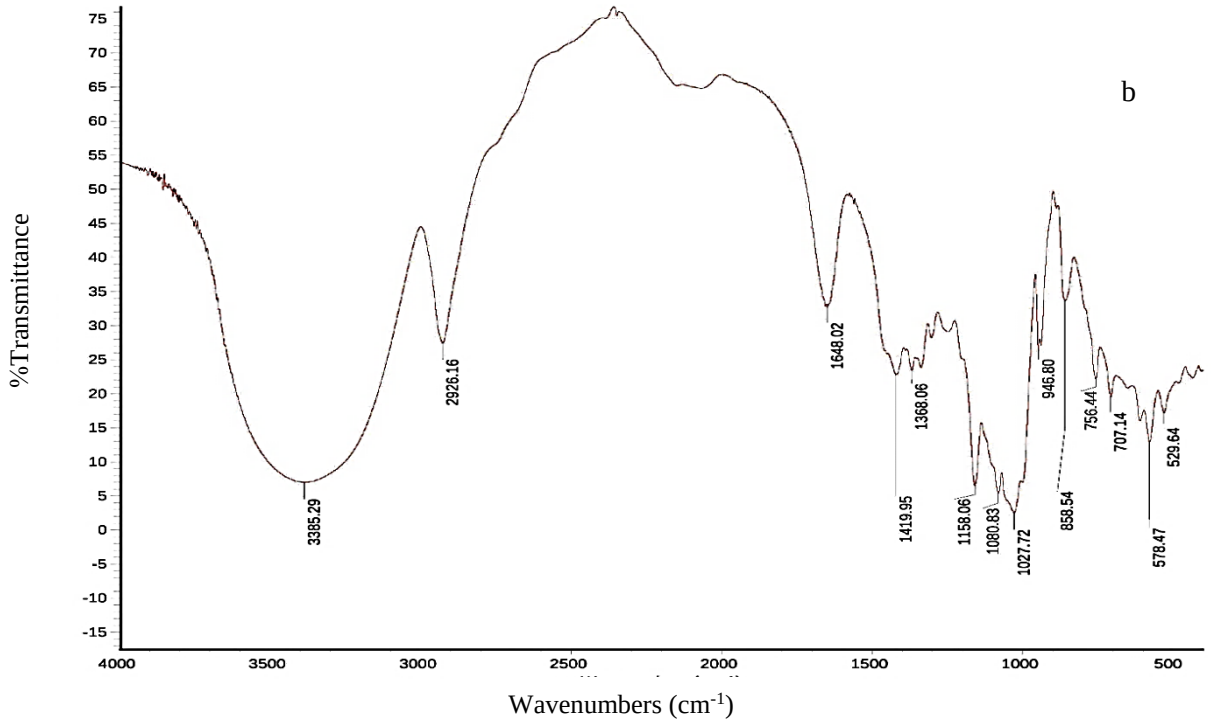


Figure 2. Pattern of particle size distributions of $W_1/O/W_2$ emulsions prepared at three ratios of $W_1/O:W_2$ and used for *Lactobacillus casei encapsulation (a) and S-shaped curves for* cumilative particle size distribution of the $W_1/O/W_2$ emulsions (b)

The span values along with volume weighted mean diameter ($D_{4,3}$) and surface mean diameter ($D_{3,2}$) are presented in Table 1. Small span values are generally indicative of monomodal droplet size distribution, and span values in the present study although appeared to be large but, monomodality observed in the graph (Fig. 2a) may be indicative of uniform distribution of dispersed phase within W_2. Essentiality of balance between hydrophilic and lipophilic emulsifier performance is the point of the interest in all of the relevant studies regarding emulsions preparation

and we believe that presence of β-CD in W_2 phase in the present study was advantageous. The values of D_{32} = 2.99 µm and $D_{4,3}$ = 6.503 µm for the emulsion prepared by the ratio of $W_1/O:W_2$ as 60:40 were lower than the relevant values for the other two prepared emulsions (Table 1). Fig. 2b also shows the particle size distribution for each of the three test double emulsions expressed as a cumulative function as S-shaped curves.





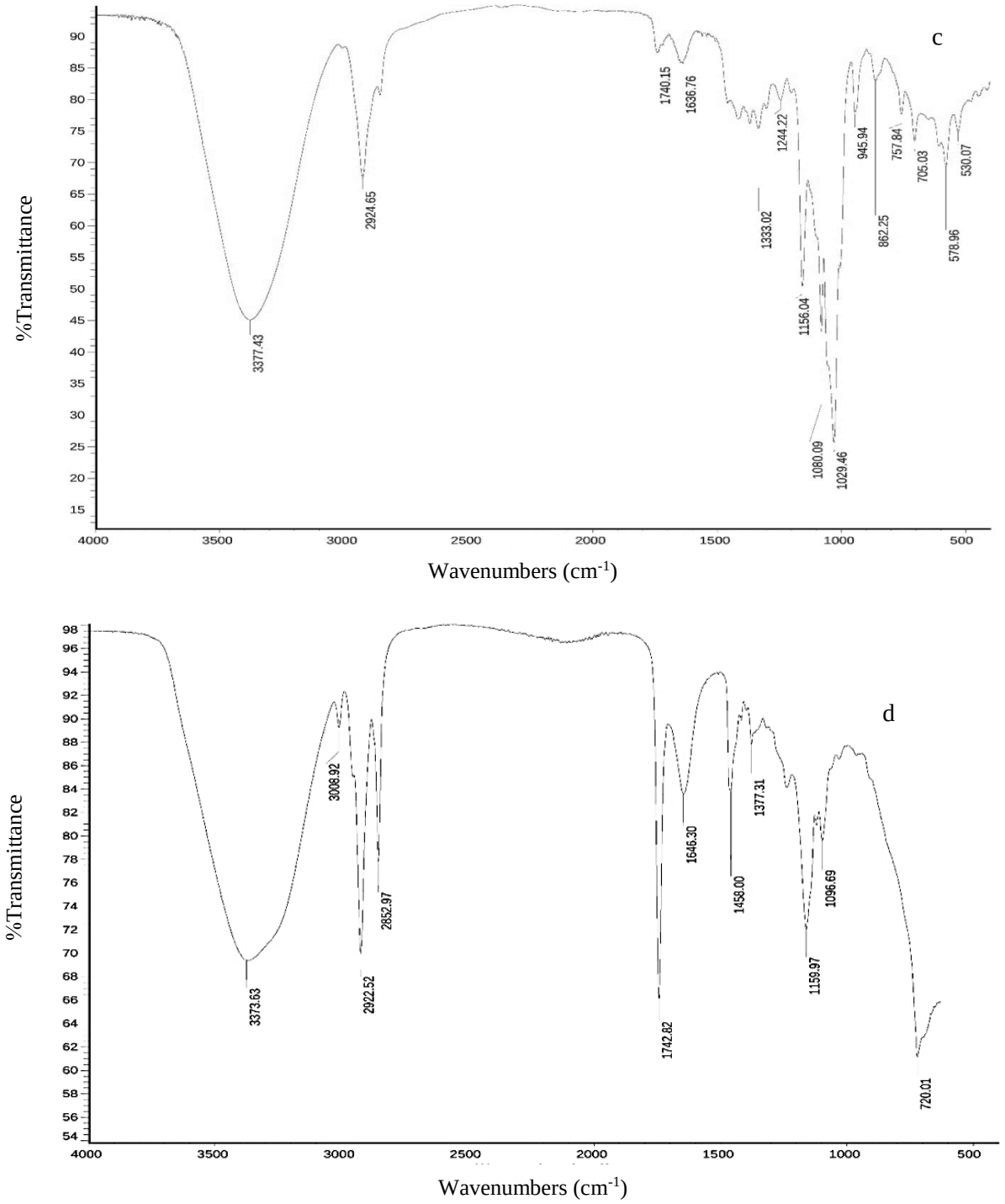


Figure 3. Fourier Transform Infrared spectroscopy spectra of *Lactobacillus casei* (a), pure form of the β-cyclodextrin powder (b), inclusion complex (IC) in the powder form (c), and $W_1/O/W_2$ double emulsion at 60:40 ratio of W_1/O: W2 (d)

Table 1. $W_1/O/W_2$ emulsion droplet mean diameter in terms of surface and volume weighed values ($D_{3,2}$ and $D_{4,3}$, respectively). Span values are also presented*.

$W_1/O:W_2$ ratio	$D_{3,2}$ (µm)	$D_{4,3}$ (µm)	$D_{0.1}$ (µm)	$D_{0.5}$ (µm)	$D_{0.9}$ (µm)	Span
40:60	3.327	8.852	1.323	5.735	21.345	3.49
50:50	3.141	7.284	1.364	4.760	17.040	3.29
60:40	2.999	6.503	1.347	4.310	14.7763	3.12

*span $= \frac{D_{0.9} - D_{0.1}}{D_{0.5}}$, $D_{0.9}$ shows diameter of 90% of the particles stay below this value and $D_{0.1}$ shows diameter of 10% of the particles stay below the given value (all these values have obtained from printout page of the Mastersizer analyzer).

Functional group characterization using Fourier Transform Infrared spectroscopy analysis was further experiments performed to describe the emulsion properties. This spectroscopy method as a valuable technique not only provides information on the functional groups of compound(s) under interest but interactions between cyclodextrin and guest molecules in these types of studies (such as present work) can also be recognized [5,16]. Fourier Transform Infrared spectroscopy spectra of the β-CD, the formed IC between β-CD and W_1/O phase and the bacterial suspension are presented in Fig. 3. In Fig. 3a, the Fourier Transform Infrared spectroscopy spectrum of the *L. casei* is presented. For the Fourier Transform Infrared spectroscopy spectrum of the β-CD, the band at 3385 cm^{-1} is representative of the vibration of symmetrical and asymmetrical stretching of the-OH groups while, the band at 2934 cm^{-1} corresponds to the vibration of C-H stretch [16]. Infrared spectroscopy is also an increasingly growing technique for the investigation of biological systems including LAB [17]. Fourier Transform Infrared spectroscopy was also employed in order to gain a further understanding of the spectral characteristics of strain *L. casei*, on bacterial suspension. Fig. 3b shows spectral characteristics of cell components such as absorption frequencies of 3257 and 1636 cm^{-1} corresponding to N-H in amide (stretching and bending) [5]. Furthermore, the Fourier Transform Infrared spectroscopy spectrum of the IC particles showed a complete difference between individual spectra of the LAB and β-CD which further confirms the formation of inclusion complex, while the peak observed at 1636 cm^{-1} in IC indicates the incorporation of LAB into the inclusion complex (Fig. 3c). Fig. 3d also presents the Fourier Transform Infrared spectroscopy spectrum of $W_1/O/W_2$ emulsion in which presence of fatty acids, amides (proteins) and phosphorlipids is confirmed [5].

Scanning Electron Microscopy images obtained in the present study provided evidence for the bacterial loading in small size capsules (Figs. 4a and 4b). The IC particles covering the interface of the W_1/O droplets are also shown in that figure.

3.2. Kinetic studies and exponential probability distribution

Two approaches were used in the present study to evaluate the decrease of cells viability: first order kinetics in the form of $- d(x) /dt = k(x)$ where, x and k represent cell count (CFU ml^{-1}) and proportionality constant (min^{-1}), respectively. The k value shows that the constant fraction of CFU ml^{-1} decreases at any given time during the experiments. Probability distribution concept describes the likelihood of an event for a random variable such as viability of the entrapped *L. casei*, using appropriate distribution. Exponential distribution was also used in the present study and survival of the entrapped cells in the model environment (gastric fluid or in an acidic medium) was monitored according to equation below:

$f(t)= \lambda e^{-\lambda t}$

Failure rate (λ) has said as the time it takes for the entrapped cells to lose the functionality (cells behavior change) and the cells would be unable to express the functionality.

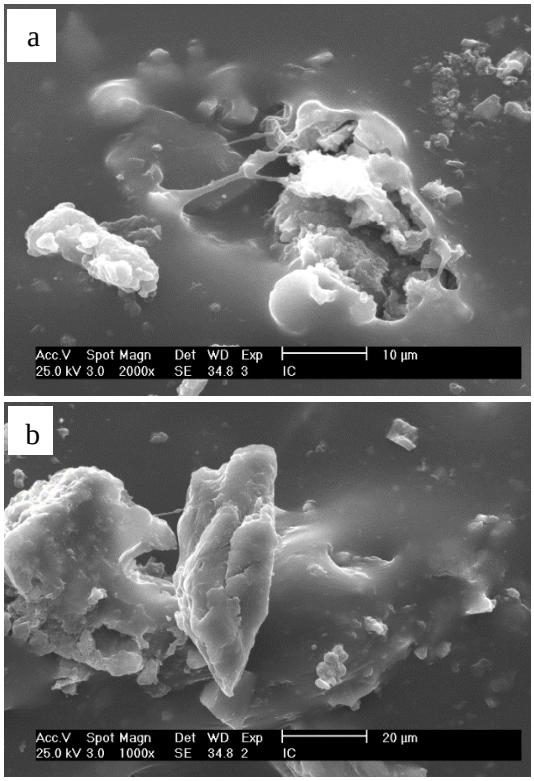


Figure 4. ESEM (Environmental Scanning Electron Microscope) images of $W_1/O/W_2$ double emulsions at 60:40 ratio of $W_1/O:W_2$, scale bar: 10 µm (a) and scale bar: 20 µm (b)

By comparing constant failure rate over time, the protective role of the Pickering type of the $W_1/O/W_2$ emulsions prepared in the present study was demonstrated.

3.2.1. *L. casei* and its bile salts and acid tolerance

Major consideration in emulsion preparation is to focus on the extent of the involved interactions between colloidal particles and between all molecules within each particle. Types of interactions can be explained as van der Waals, electrostatic, steric and hydrophobic interactions, etc. [8]. The balanced forces and interactions could ultimately describe emulsion stabilization. Cyclodextrin as the enzymatic derivative of starch is used in the present study as a Pickering emulsion stabilizer (i. e., emulsifier) which acts by producing a complex with oil phase content at oil-water interface, so stabilizes the test emulsions [10]. Different instrumental analyses provide evidence for complexation processes and its extent [12,13,18]. Besides β-CD, Span 80 was the emulsifier used in the preparation of the primary emulsion in the present study. The nature

and amount of emulsifier actually affect the passage of water from W_1 and from W_2, as the outer aqueous phase in the double emulsion network is affected by the emulsifier nature and demand and limited water transportation thus provides a favorable condition in terms of *L. casei* as the core bioactive compound [19].

Survivability of *L. casei* within $W_1/O/W_2$ emulsion according to the kinetic results is presented in Figs. 5a and 5b. The k constant values which are calculated simply from the slope of the line (s) shown in Fig. 5, are given in Tables 2a and 2b. In the present study, the efficiency of emulsification strategy in keeping *L. casei* cell viable has been determined and the decreasing trend of k constant values shown in Table 2 confirms the extent of tolerance of *L. casei* in bile salts and acidic pH environment (s). Further note on the efficiency of the test double emulsions in keeping viability of the entrapped *L. casei* is to examine $t_{1/2}$ values as the time to decrease the viable cells count by 50% of the original count which are shown in Table 2. The $W_1/O/W_2$ prepared at the ratio of 60:40 responded favorably to bile salts presence in the test medium compared to the control (dispersing the bacterial cells directly in the test medium) as shown in Table 2 ($(t_{1/2})_{60:40} > (t_{1/2})_{control}$). Additionally, relative viability curve showed about 30% of the cells in control sample were viable after test time, while the percentage of cell survival for W_1/O: W2 at 60:40 ratio was 70% (Fig. 5, inset). Besides, regarding the relative viability curve the acid tolerance of the *L. casei* entrapped within the double emulsion (ratio of 60:40) was observed. Although the system was protective for the entrapped cells, 53% of the cells remained viable after 3 h in the acid medium.

Table 2. The calculated values of rate constant (k) and half-life ($t_{1/2}$) for the first order kinetic model used to study entrapment of *Lactobacillus casei* within $W_1/O/W_2$ emulsion where the double emulsions were incubated with either the bile salts-0.3 % (a), or in the acidic environment-pH 2 (b)

(a)

$W_1/O:W_2$ ratio	k (min^{-1})	t ½ (min)
60:40	0.002 (0.9604)	346.5
50:50	0.003 (0.912)	231
40:60	0.003 (0.9432)	231
Control system	0.005 (0.9223)	138.6

(b)

$W_1/O:W_2$ ratio	k (min^{-1})	t ½ (min)
60:40	0.003 (0.9691)	231
50:50	0.004 (0.9567)	173.25
40:60	0.004 (0.9667)	173.25
Control system	0.006 (0.9479)	115.5

The calculated values of coefficient of determination are shown in parentheses.

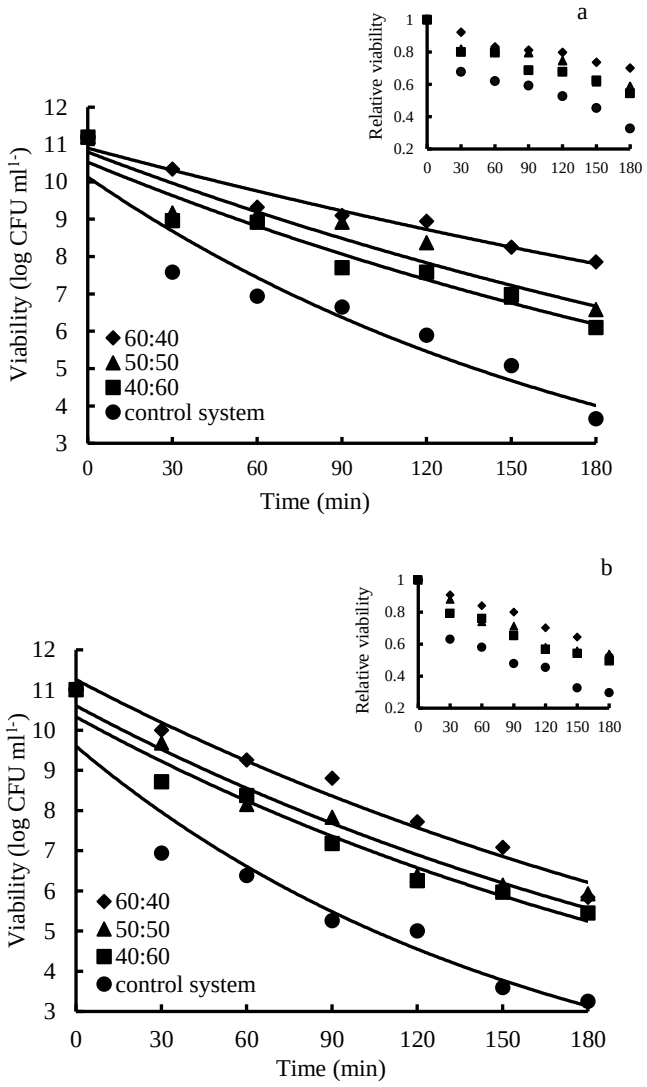


Figure 5. Application of first-order kinetic describing viability of the entrapped *Lactobacillus casei* within the $W_1/O/W_2$ emulsions: trend of cells viability when the encapsulated cells were incubated in the medium containing bile salts at 0.3% concentration (a) and in an acidic medium (pH 2) (b) (The curves for relative cells viability versus time are given as inset.)

In addition to proportionality constant of k, the kinetic approach is extended and the results in terms of $t_{1/2}$ would be similarly interpreted (Table 2). The result reported by Shima et al. and without kinetically handling data, confirms the protectiveness of $W_1/O/W_2$ emulsion for *L. acidophilus* [20]. Argin et al. also investigated the effectiveness of xanthan-chitosan hydrogels in protecting probiotics under simulated gastric fluid with use of cell release kinetics from microcapsules. The results showed that using xanthan-chitosan as the core material increased the viability rate of probiotic bacteria in harsh conditions [21].

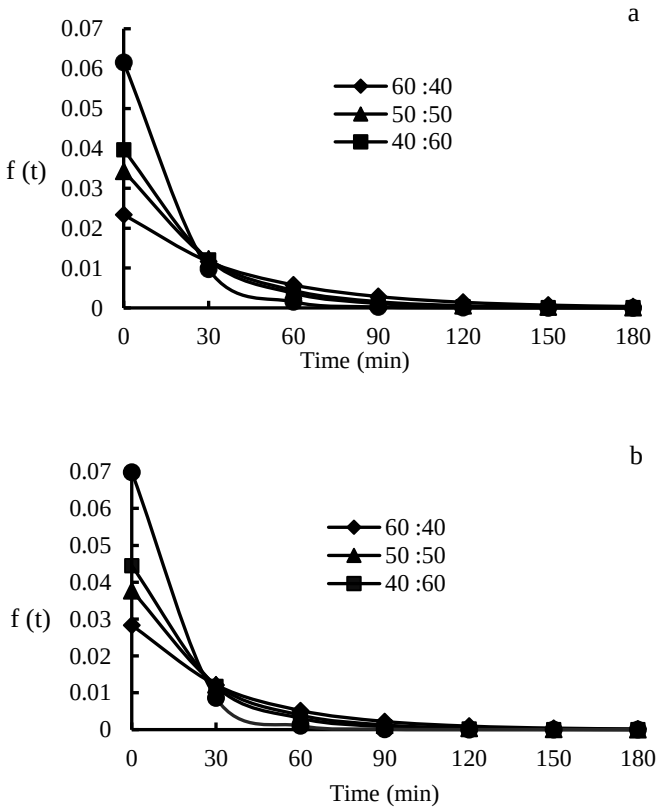


Figure 6. Use of the exponential probability distribution model ($f(t)=\lambda e^{-\lambda t}$) for describing the tolerance of the entrapment of *Lactobacillus casei* within the $W_1/O/W_2$ emulsions in the bile salts medium (a) and in the acidic medium (pH 2)(b)

Another approach undertaken by Shima et al., was based on using Weibull distribution when the researchers could not obtain a straight line from semi-log plot of the survival rate against time [22]. The Weibull distribution has widely been used in the reliability analysis such as studying bacterial inactivation [23-25]. The focusing point in the present study was to treat the data kinetically (first order reaction rate) and also probabilistically (exponential distribution).

In determining bile salt and acid tolerances of the entrapped cells, another approach used in the present study was to apply exponential probability distribution. The results in Tables 3a and 3b provide insights into how probable it is for the entrapped cells to remain viable after 30 min (in response to either bile salt or acidic environment).

Considering the cells performance in bile salt environment, 42% of *L. casei* entrapped in double emulsions (60:40 ratio) were viable after the first 30 min, while 1% of the cells in the control system remained alive during the same period. In other words, the free cells viability in bile salts medium after 150 min was negligible and in very short time, the cells lost their functionality Tables 3a and 3b shows the following decreasing order of the constant failure rate for the encapsulated *L. casei*: 40:60<50:50<60:40. The cells remained viable for longer time in the emulsions prepared at 60:40 ratio of W_1/O: W2 and the other two ratios used for the emulsion preparation were considerably less protective for *L. casei* entrapment. Further note is on Fig. 6 which was drawn to show the trend of use of the exponential probability distribution model. Preference of cells response to bile salt (Fig. 6a) and acidic environments (Fig. 6b) when entrapped in double emulsions (60:40 ratio) is understandable and it means probabilistically lower viable cells remained within the emulsion. In other word, most of the viable cells were released.

Table 3. Application of probability distribution concept in the present study: exponential failure time distribution used to describe the event for the double emulsion incubated either in bile salts -0.3% (a) or in the acidic environment-pH 2 (b)(a)

$W_1/O:W_2$ ratio	Time (min)						
	0	30	60	90	120	150	180
60:40	0.424759	0.153135	0.122666	0.10444	0.052304	0.035176	0.153135
50:50	0.13116	0.124343	0.102327	0.058826	0.013703	0.009864	0.124343
40:60	0.107006	0.10179	0.030207	0.026813	0.014752	0.006107	0.10179
Control system	0.026813	0.014064	0.010512	0.004959	0.0022	0.000529	0.014064

(b)

$W_1/O:W_2$ ratio	Time (min)						
	0	30	60	90	120	150	180
60:40	0.363092	0.173219	0.110549	0.03715	0.019655	0.005686	0.363092
50:50	0.265139	0.05712	0.041748	0.00977	0.007707	0.006225	0.265139
40:60	0.101004	0.071791	0.021779	0.008624	0.006443	0.003843	0.101004
Control system	0.017091	0.009753	0.003171	0.002458	0.000601	0.000426	0.017091

3.2.2. Cell viability as a function of heat Treatment

Pasteurization is considered as a common process in food and beverage industries for deactivation of pathogenic microorganisms. The temperature needed to preserve different types of foods from spoilage by organisms and enzymes is around 65°C [4,26]. However, to benefit positive effects from probiotic bacteria on human body, the survival of LAB during heat treatment is an important factor. In the present study, Fig. 7 presents the viability of the free and entrapped *L. casei* in $W_1/O/W_2$ double emulsion under the heat treatment at 65°C for 15 and 30 min. As it is shown, after 30 min exposure to the temperature of 65°C, more than 50% of free cells died which shows their sensitivity to the elevated temperature. The $W_1/O/W_2$ emulsion technique has provided a matrix to enhance the bacterial resistance against high temperatures by inhibiting the heat diffusion into the W_1 phase where the *L. casei* is entrapped. Furthermore, W_1/O: W2 ratio of 60:40 showed better thermal stability and a greater number of cells remained viable after both 15 and 30 min. In a study by Yao et al. on thermal stability of the free and entrapped *L. salivarious Li01* at 65°C, the entrapped *L. Li01* within alginate/gelatin-microgels showed high resistance and the cells remained viable, while almost all the free cells died after 30 min [4]. In another study, four different strains of probiotic bacteria (including *L. casei*, *Bifidobacterium bifidum*, *L. rhamnosus*, and *Bifidobacterium adolescentis*) encapsulated by alginate-Hylon starch microcapsules with genipin cross-linked chitosan and poly-L-lysine, showed more resistance against high temperature (72, 85, and 90˚C) after 30s [27]. Ding et al. also claimed that microencapsulation can improve the resistance of probiotic bacteria in mild heat treatment. However, both free and protected bacteria lost their viability after 60 min of heating [28].

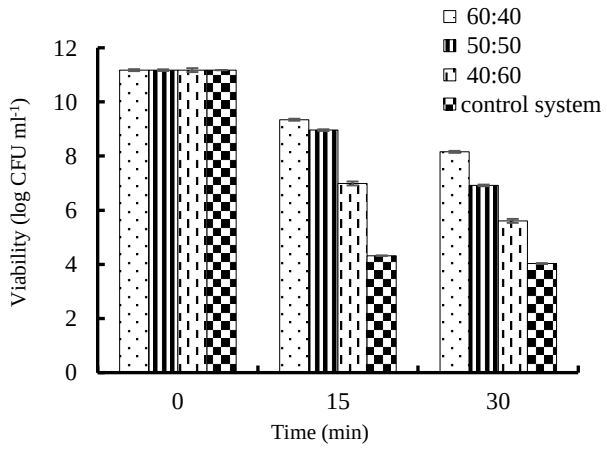


Figure 7. Viability of the encapsulated cells in response to heat treatment (65°C)

4. Conclusion

This study showed that β-CD inclusion complexes may be used to produce micron-sized Pickering-type $W_1/O/W_2$ emulsions for entrapping *L. casei*. In addition to the structural characterization of the double emulsions (Fourier Transform Infrared spectroscopy, size distribution and Scanning Electron Microscopy), the release of the entrapped cells was kinetically described. Further explanation regarding the release of the viable cells was based on probability distribution function (exponential distribution). The most stable emulsions containing the highly viable LAB were prepared at $W_1/O:W_2$ ratio of 60:40 and these emulsions were resistant against high temperature (65°C) during 30 min. It would be appropriate to extent study on kinetic release by applying different patterns of probability distribution as well.

Encapsulation of probiotic bacteria using emulsification technique in the form of $W_1/O/W_2$ emulsions having edible composition (including β-CD emulsifier) provides an acceptable procedure for practical use. In-vitro studies (by preparing bile salts) have directed experiments to simulate conditions of gastro-intestinal tract and the results of the release kinetics were favorable and confirmed efficiency of emulsification as an encapsulation process. The applied technique in the present work was reliable and further in-vivo studies are needed to support usage of the findings in industry.

5. Acknowledgements

The authors would like to thank Amirkabir University of Technology and Materials and Energy Research Center for their financial supports.

6. Conflict of interest

The authors report no conflicts of interest.

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Research Article

APPLIED FOOD BIOTECHNOLOGY, 2020, 7 (3): 171-182
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

ریزپوشانی *لاکتوباسیلوس کازئی* در فاز درونی امولسیون آب/روغن/آب ($W_1/O/W_2$) تهیه شده با بتا سیکلودکسترین و بررسی زنده‌مانی باکتری در سامانه مدل معده

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چکیده

سابقه و هدف: در متون علمی تحقیقات گسترده‌ای در خصوص استفاده از امولسیون‌های دوتایی در حوزه‌های مختلف از قبیل مواد غذایی، دارویی و محصولات بهداشتی صورت گرفته است. در تحقیق حاضر، امولسیون دوتایی آب در روغن در آب ($W_1/O/W_2$) برای ریزپوشانی *لاکتوباسیلوس کازئی* به‌کار گرفته شده است، در حالی که تعادل سامانه معده تحت تاثیر مکانیسم رهایش زیست‌یارهاست. نقش امولسیفایری بتا سیکلودکسترین (β-CD) در فاز آبی خارجی امولسیون با آنالیز ویژگی‌های امولسیون (طیف بینی مادون قرمز تبدیل فوریه، توزیع اندازه ذرات و میکروسکوپ الکترونی روبشی) قابل استناد به نظر می‌رسد. احتمال زنده‌مانی سلول‌ها در سامانه معده با استفاده از مدل کینتیکی درجه اول و مفهوم احتمال و تابع توزیع نمایی بررسی شد.

مواد و روش‌ها: سه نسبت متفاوت $W_1/O:W_2$ (۴۰:۶۰، ۵۰:۵۰ و ۶۰:۴۰) برای تهیه امولسیون‌های $W_1/O/W_2$ به منظور ریزپوشانی *لاکتوباسیلوس کازئی* در فاز داخلی با استفاده از امولسیفایر β-CD مورد استفاده قرار گرفته است. با استفاده از روش پراکنش دینامیکی نور اندازه ذرات امولسیون تعیین گردید. حضور گروه‌های عاملی از طریق طیف‌سنجی مادون قرمز و ثبت پیک‌های مربوطه در محدوده $4000-400\ cm^{-1}$ مشخص گردید. معادله دیفرانسیلی سینتیکی درجه اول ($dx/dt=kx$) برای تعیین زنده‌مانی سلول‌ها مورد استفاده قرار گرفت. از تابع توزیع نمایی احتمال برای نشان دادن زمان شکست (بیانگر سلول‌هایی که قادر به رها شدن نیستند) استفاده شد ($f(t)=\lambda e^{-\lambda t}$).

یافته‌ها و نتیجه‌گیری: با مدل‌سازی سامانه معده با استفاده از نمک‌های صفراوی و محیطی با pH تنظیم شده، زنده‌مانی سلول‌های ریزپوشانی‌شده پایش شد و ثابت معادله کینتیکی درجه اول به دست آمد ($k(60:40) < k(50:50) < k(40:60)$). به نظر می‌رسد از آنجا که امولسیون‌های مورد استفاده برای ریزپوشانی *لاکتوباسیلوس کازئی* کاملا خوراکی است، امولسیون‌سازی از نظر صنعتی امکان‌پذیر می‌باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۱ فوریه ۲۰۲۰

داوری ۱۹ می ۲۰۲۰

پذیرش ۳۱ می ۲۰۲۰

واژگان کلیدی

- توزیع نمائی
- محیط معده
- مدل کینتیکی
- زنده‌مانی *لاکتوباسیلوس کازئی*
- امولسیون دوتایی آب در روغن در آب ($W_1/O/W_2$)

***نویسنده مسئول**

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