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Emulsion stabilization by tomato seed protein isolate: Influence of pH, ionic strength and thermal treatment

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Abstract

There is a burgeoning interest for plant protein-based emulsifiers owing to their economic benefits and lower environmental impacts. This study investigated the stability of 10.0 wt% oil-in-water emulsions stabilized by 1.0 wt% protein extracted from tomato seeds, a by-product of tomato processing industries. Both water-soluble albumin and salt-soluble globulin fractions of tomato seed protein in the molecular weight range of 48-10 kDa were adsorbed at the oil-water interface, as confirmed by sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE). Tomato seed protein isolate-stabilized emulsions were subjected to environmental stresses such as pH (2-9), ionic strength (0-250 mM NaCl or $CaCl_2$) and thermal treatment (30-90°C, 30 min). Droplet size, droplet charge, microstructure and creaming stability were assessed. Emulsions were stable to droplet aggregation except at pH 2-4, owing to their proximity to isoelectric point. Emulsions showed excellent stability to high NaCl concentrations (250 mM) at pH 6-8 with surface charges above -40 mV. However, extensive droplet flocculation with aggregated optical microstructure and creaming indices was observed in presence of $\geq$ 50 mM $CaCl_2$, which was attributed to ion binding and charge screening effects. Droplet aggregation above 80 °C was due to the denaturation of the globular protein fractions adsorbed at the interface. These results might have implications for the utilization of tomato seed protein as potential emulsifier for food and beverage applications.

Keywords

Tomato seed protein, salt stability, SDS-PAGE, thermal denaturation, creaming, charge screening

Introduction

Dairy proteins such as caseins and whey proteins are ubiquitously used as natural emulsifiers in food industries because of their abilities to adsorb to oil-water interfaces during homogenization and kinetically retard droplet aggregation by forming protective coating around the oil droplets (McClements, 2004; Ozturk & McClements; Singh, 2011; Wilde, 2009). However, due to the rising commodity prices of dairy ingredients, current trends of "dairy-free" and "vegan" labelling and increasing debate on environmental footprints of dairy protein production (Day, 2013), plant protein alternatives are gradually receiving increased research and industrial attention. Besides soy protein, recent literatures pea protein (Liang & Tang, 2013), amaranth (Ventureira, Martínez, & Añón, 2010), potato (Romero, et al., 2011) proteins have shown their potential for valorisation as emulsifiers. Interestingly, agricultural by-products can also be a potential source to widen the basket of novel proteins, which has gathered relatively limited attention so far. For instance, huge amounts of wastes such as fruit seeds are produced during the industrial processing of fruits and vegetable based food products. If the functionalities of protein extracted from those by-products are proven, utilization of such protein can not only contribute to economic benefits being a low cost emulsifier but also help to reduce solid waste.

Tomato (Lycopersicon esculentum L.) is a well-known fruit worldwide with annual production at 100 million tons (Kalogeropoulos, Chiou, Pyriochou, Peristeraki, & Karathanos, 2012). Extensive use of tomatoes as finished products such as tomato sauce, juice, ketchup, puree, powder or as processed ingredients in prepared foods such as pizza, pasta, snacks, generates large quantities of solid wastes during the manufacturing process. These by-products mainly comprise of peel and seeds, which can result in land fill and associated environmental issues (Shao, et al., 2014; Sogi, Bhatia, Garg, & Bawa, 2005). Although tomato peel has been significantly studied as a source of value-added bioactive

such as lycopene and β-carotene (Kalogeropoulos, et al., 2012; Lavecchia & Zuorro, 2010; Papaioannou & Karabelas, 2012; Rozzi, Singh, Vierling, & Watkins, 2002), tomato seeds, even being 60 % of the total by-product have attracted very limited attention. Tomato seeds have been reported to contain approximately 25-30% of crude protein and are highest in glutamic acid and aspartic acid (Persia, Parsons, Schang, & Azcona, 2003). Unlike many other plant proteins, tomato seed has been also reported to have a high lysine content (Brodowski & Geisman, 1980; Sarkar & Kaul, 2014). Tomato seeds have also been reported to be a better source of protein as compared to other alternative sources since there are no anti-nutritional factors or harmful constituents found in tomato seeds (Sogi, et al., 2005). A recent study has shown its functional properties in terms of emulsifying and foaming capacities (Shao, et al., 2014).

Emulsion-based products typically experience a variety of conditions during manufacturing such as presence of salts and acids in the formulation. Protein-stabilized emulsions tend to be particularly sensitive to ionic strength and temperature. Emulsions generally tend to flocculate when the ionic strength exceeds a particular level, because the net repulsive electrostatic forces between the droplets are relatively weak to overcome the various attractive interactions (Tokle & McClements, 2011). Even after consumption, emulsions are exposed to various pH and ionic environments depending upon the respective site from mouth to intestine (Sarkar, Goh, & Singh, 2010; Singh & Sarkar, 2011). Furthermore, emulsions stabilized by globular proteins are particularly sensitive to thermal treatments as there is the possibility of the proteins to unfold and expose reactive groups originally located within their hydrophobic domains (McClements, 2004). Denaturation of the protein at the oil-water interface and interaction between proteins coated to different droplets might take place depending upon heating, which may eventually result in droplet aggregation.

For tomato seed proteins to be used as an emulsifier, it is therefore important to understand the influence of pH change, presence of salts such as Na^{+}, Ca^{2+} ions, heat treatment conditions such as pasteurization on the physicochemical properties and stability of tomato seed protein-stabilized emulsions. To the best of our knowledge, no published research report exists that has systematically investigated the stability of emulsions stabilized by tomato seed protein isolate when subjected to environmental stresses such as pH, ionic strength, and temperature changes. Hence, the objective of this study was to stabilize sunflower oil-in-water emulsions using protein isolated from tomato seeds and to establish the influence of major factors (pH, ionic strength, and temperature conditions) on the stability of tomato seed protein-coated droplets. The insights generated in this study can be useful for determining the range of pH, temperature and ionic strength conditions where tomato seed protein can be successfully employed as an emulsifier.

Materials and Methods

Materials

Air-dried tomato seeds (6-8% moisture content) were purchased from E W King & Co Ltd, Essex, UK. Sunflower oil was purchased from local supermarket (Morrisons, UK). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) chemicals were purchased from Bio-Rad, UK. All other chemicals were of analytical-grade and purchased from BDH Chemicals (BDH Ltd, Poole, UK) unless otherwise stated. Milli-Q water (water purified by a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) was used as a solvent in all experiments.

Tomato seed protein isolate (TSP) preparation

Tomato seed protein isolate was obtained from dried tomato seeds using salt extraction and pH based protein precipitation technique described previously (Sarkar, et al., 2014). Briefly, the dried raw tomato seeds were ground using a hammer mill (Christy and Norris, Essex, UK) with screen size of 0.8 mm to create a fine powder. Tomato seed powder (40 g) was extracted for 1 hour with 400 mL of 1M NaCl solution at 50 °C at pH 8, adjusted using 0.1N NaOH. The resulting slurry was centrifuged at 8000 rpm for 30 minutes using Avanti J-30I centrifuge (Beckman Coulter, USA) and the supernatant was collected. The pH of the supernatant was adjusted to the isoelectric point (pH 3.5) using 0.1 N HCl and the precipitate was separated by centrifugation at 8000 rpm for 30 min. The supernatant obtained was neutralized and dialyzed using dialysis tubings of 6000-8000 molecular cut off (M/s. Thomas Scientific Co., Philadelphia USA) immersed in Milli-Q water overnight to remove excess NaCl in refrigerated condition (4 °C). The solution was freeze dried using freeze dryer (Christ Alpha 1–4 Lyophilizer, Osterode, Germany) at a temperature of -50°C for 48 hours to obtain tomato seed protein isolate (TSP). The resultant TSP contained 91.8% protein as determined by Kjeldahl method (AOAC, 1995), which is in line with the results of previous study (Sarkar, et al., 2014).

Emulsion preparation

The TSP (1.0 wt% protein) solution was prepared in 10 mM phosphate buffer by stirring for 2 h at 20 °C. The pH of the solution was adjusted to pH 7.0 using 1 M NaOH or 1 M HCl. An emulsion was prepared by mixing appropriate quantities of TSP solution (90 wt%) and sunflower oil (10 wt%). The mixture of sunflower oil and protein solution was pre-emulsified using a conventional rotor-stator type mixer (L5M-A, Silverson machines, UK) at 7000 rpm for 2 minutes. The sample was then homogenized by two passes using a two-stage valve high

pressure homogenizer (Panda Plus 2000, GEA Niro Soavi - Homogeneizador Parma, Italy) operating at 250 bars for the first stage and 50 bars for the second stage. The emulsion samples were prepared in triplicates. Sodium azide (0.02 wt%) was added to prevent microbial growth during storage of the emulsions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

The protein composition of the TSP solution as well as the adsorbed TSP at the oil-water interface was analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Freshly prepared emulsion was centrifuged for 20 min at 4200 g at 20 °C. The cream layer was carefully removed, dispersed in Milli-Q water and again centrifuged for 20 min at 4200 g at 20 °C. The cream layer was collected carefully and a certain amount of cream was spread on to a filter paper (Whatman No. 42, Whatman International Ltd., Maidstone, Kent, UK) and dried. The dried cream containing the adsorbed TSP was then mixed with SDS buffer (0.5 M Tris, 2.0% SDS, 0.05% β-mercaptoethanol, pH 6.8) (sample: sample buffer = 50 μg:150 μL, and heated to 95-100 °C for 5 min. The TSP protein solution was also mixed with SDS buffer as indicated above. SDS-PAGE was carried out by loading 10 μL of samples on to the gels previously prepared on a Mini-PROTEAN II system (Bio-Rad Laboratories, Richmond, CA, USA). The resolving gel contained 16.0% acrylamide and the stacking gel was made up of 4.0% acrylamide. After running, the gel was stained for 45 min with a Coomassie Brilliant Blue R-250 solution and 20% isopropanol. The gels were destained with a solution of 10% acetic acid and 10% isopropanol and scanned using a Gel Doc™ XR+ System (Bio-Rad Laboratories, Richmond, CA, USA).

Influence of pH, ionic strength and thermal treatment

The prepared emulsions were kept at room temperature for 24 hours before any adjustments of pH, ionic strength or temperature.

pH stability

The pH of emulsions was altered to create a series of samples adjusted to pH 2-9 by adding either 0.01–2 M HCl or NaOH under continuous stirring for 1 hour at 500 rpm using a magnetic stirrer.

Salt stability

The influence of ionic strength on emulsion stability was determined by adding different amounts of either NaCl or $CaCl_2$ salts in powdered form to the freshly prepared emulsions (non pH adjusted) to get the desired salt concentrations (25 mM to 250 mM) under stirred conditions at 500 rpm using a magnetic stirrer. The emulsions were stirred thoroughly for 1 hour to ensure complete dissolution of the salts. The pH of the emulsions were measured and then adjusted to pH ranging from pH 2-8 by adding either 0.01–2 M HCl or NaOH under continuous stirring conditions. These emulsions were then stored overnight at room temperature before being analyzed.

Temperature stability

The influence of temperature on emulsion stability was examined by placing the emulsions in individual glass test tubes, incubating them in a water bath set a fixed temperature (30-90°C) for 30 min at pH 7, and then cooling them to room temperature by placing the tubes in ice.

Droplet size determination

Mean hydrodynamic diameter (Z-average) of emulsions was determined by dynamic light scattering (DLS) with the detector at 173° (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, UK). Emulsion samples were diluted at a ratio of 1:100 (v/v) in pH adjusted Milli-Q water and then placed in a cuvette before measurement. Each sample was run five times; each run consisted of three acquisitions that lasted 60 seconds/acquisition. Result was reported as the mean and the standard deviation calculated of the five readings from an individual sample.

It should be noted that DLS shows the hydrodynamic diameter of particles based on the assumption that the particles are isolated homogeneous spheres. In case of aggregated emulsions, the droplets flocculate into non-spherical and heterogeneous particles and the light scattering gives an approximate indication of the hydrodynamic diameter of these flocs. Hence, the results particularly in aggregated samples should be treated with some caution. A supplementary information sheet of correlogram and size distribution has been provided for samples with large hydrodynamic diameter (> 2000 nm).

Optical microscopy

The microstructure of the emulsions was investigated using standard transmission light microscopy. The emulsion samples were placed on a clean dry microscope slide and then covered with a coverslip. Light microscopy images were taken using a Nikon Optishot (Nikon, Japan) microscope with an Olympus SLMPlan using × 40 magnification.

ζ-potential measurements

Zeta-potential (ζ-potential) was determined by laser doppler velocimetry and phase analysis light scattering (M3-PALS) technique (Zetasizer Nano ZS series, Malvern Instruments,

Worcestershire, UK). One millilitre of diluted sample (diluted to approximately 0.001 wt% droplet concentration) was put into a folded capillary cell (Model DTS 1070, Malvern Instruments Ltd., Malvern, Worcestershire, UK). An individual ζ-potential measurement was calculated from the mean and the standard deviation of at least ten readings from an individual sample.

Determination of creaming stability

The emulsions were transferred into 2 mL transparent plastic tube that was tightly sealed with a plastic cap. After seven days of storage, the extent of creaming was assessed using creaming indices (Wu, et al., 2012), which was defined as $CI_C = 100 \times \left(\frac{H_C}{H_E}\right)$ for the cream layer and $CI_S = 100 \times \left(\frac{H_S}{H_E}\right)$ for the serum layer, where H_E is the total height of the emulsion, H_S is the height of the subnatant (turbid) serum layer and H_C is the height of the opaque cream layer. Photographs of emulsion samples were taken over a period of 7 days using a digital camera to record the droplet creaming.

Statistical analyses

The results were statistically analyzed by analysis of variance (ANOVA) using Graphpad 5 Prism software and differences were considered significant when $p<0.05$ were obtained.

Results and discussion

Composition of TSP at the interface

To determine the protein composition, SDS-PAGE of the TSP protein solutions (1 wt%) and TSP adsorbed at the oil-water interface under reducing conditions is shown in Figure 1. Analyses of the SDS-PAGE electrophoreogram of TSP solution revealed four main protein

fragments with molecular weights of 48 kDa, 33 kDa, 20 kDa, 19 kDa and 10kDa, extracted in the protein isolate using the salt and alkali precipitation technique (Figure 1). This is broadly in agreement with previous work (Sogi, Arora, Garg, & Bawa, 2002), where water-soluble (albumin) and salt-soluble (globulin) fractions of tomato seed protein in the molecular weight range of 48-19 kDa were identified. The 10 kDa band observed in our study was not seen in previous study, which might be due to the 10% resolving gel used in the previous study unable to hold such smaller protein fragments. Interestingly, the analysis of the TSP present at the oil-water interface also resolved into a similar pattern of protein fragments. This indicates that all the extracted albumin and globulin fractions had the ability to adsorb onto the emulsion droplet.

Effect of pH on the properties of TSP-stabilized emulsions

The sunflower oil-in-water emulsion (10 wt%) stabilized by 1 wt% tomato seed protein (TSP) had an even distribution of finely dispersed droplets as characterized by optical micrograph, and Z-average diameter of 533 nm at pH 7 (Figure 2). The visual observation showed no phase separation during the seven days of storage period (data not shown). The TSP-stabilized emulsion droplets were anionic (-43.1 mV) at pH 7, which is in line with the value reported by a previous study (Velev, et al., 1993). At neutral pH, tomato seed protein forms a strong viscoelastic film at the oil-water interface supported by intra-droplet hydrophobic interaction (Kiosseoglou, Theodorakis, & Doxastakis, 1989). It is known that the pH of the emulsion-based foods and beverages vary significantly depending upon the kind of the food products in which the oil droplets are present. And, the stabilization of emulsions against coalescence and flocculation is largely dependent on the repulsive forces between the protein films adsorbed onto the emulsion droplets (McClements, 2004), which might alter based on pH change. We therefore examined the impact of change in pH on the

physicochemical properties and microstructure of TSP-stabilized emulsion (Figure 2).

At pH 2-4, the droplet size for emulsions became relatively large with highest droplet size (8346 nm) at pH 3. At pH 2, the optical micrograph showed some degree of floc formation (Figure 2A). This was further enhanced at pH 3 showing extensive droplet aggregation (Figure 2B). Between pH 2-4, the zeta-potential value ranged between +16.7 mV and -12.5 mV. Such low magnitude of absolute ζ-potential value might be attributed to the emulsions being at or near to the isoelectric point of TSP, which would mean surface neutrality i.e. the number of positively charged amino groups being balanced by the number of negatively charged carboxyl groups at the surface. Therefore the electrostatic repulsion of the TSP adsorption layers were ineffective to prevent the droplets from aggregating together (Tcholakova, Denkov, Sidzhakova, Ivanov, & Campbell, 2005) as observed clearly in the optical micrograph (Figure 2C). Similar behaviour has been reported in oil-in-water emulsions stabilized by other plant proteins, such as soy, pea, potato, amaranth, lupin (Liang, et al., 2013; Raymundo, Sousa, & Empis, 2000; Romero, et al., 2011; Ventureira, et al., 2010; Yin, Deng, Xu, Huang, & Yao, 2012) as well as milk proteins (Demetriades, Coupland, & McClements, 1997; Surh, Decker, & McClements, 2006) at the pH close to their isoelectric points. The instability of TSP-stabilized emulsions close to the pI of TSP corresponds to the minimum solubility of the stabilizing proteins themselves and their minimum effectiveness as emulsifiers. The lowest ζ-potential values recorded at pH 3 is generally consistent with highest droplet size and extensive aggregation in micrograph indicates that the pI of the TSP at the interface was in that region. The isoelectric point of the TSP protein solution was identified to be pH 3.5 based on zeta potential measurements and solubility curve of protein solutions (not shown), which is in agreement with the range reported in earlier studies (Kramer & Kwee, 1977; Liadakis, Tzia, Oreopoulou, & Thomopoulos, 1995; Shao, et al., 2014). The minor discrepancy between pIs could be attributed to the possible difference

between TSP being in an aqueous phase (in case of solution) as compared to being at oil-water interface. During homogenization, the possible unfolding of the globulin fractions and adsorption of some charged moieties to the droplet surfaces might have resulted in such shift of pI for TSP stabilized interface as compared to that of TSP in solution.

At pH 5 and above, the mean droplet remained small (509-563 nm), which was also supported by the uniformly dispersed microstructure with no evidence of droplet aggregation (Figure 1C). The magnitude of net negative charge on the protein increased significantly ($p < 0.05$) to stabilise the droplets electrostatically against aggregation as a function of pH above pH 5. As expected, above the pI, the carboxyl groups became negatively charged and the amino groups were neutral which in turn caused the droplets to gain a net negative charge. Hence, the most likely stabilization mechanism preventing droplet aggregation is electrostatic repulsion in TSP-stabilized emulsions. It is also worth noting that TSP has high proportion of acidic amino acids, glutamic acid and aspartic acid that are ionisable at high pH values (Sarkar, et al., 2014). These results suggests that TSP has good potential to be used for the emulsion formulation at neutral conditions, unlike other plant proteins such as pea protein which shows better emulsion stability at pH 3 where the former is most unstable (Liang, et al., 2013).

Effect of NaCl on the properties of TSP-stabilized emulsions

The influence of salt concentration on TSP-stabilized emulsion stability was investigated at pH values below the pI (pH 2), close to the pI (pH 4), and above the pI (pH 6 and pH 8). Figures 3A and 3B show the droplet size and zeta potential of the TSP-stabilized emulsions as a function of ionic strength (25 to 250 mM NaCl) at a pH range of 2 to 8. Interestingly, the droplet size of the emulsion did not vary significantly with increasing concentration of NaCl at pHs 6 and 8 and remained below 600 nm *($p > 0.05$)*. However, at pHs 2 and 4, the droplet

size increased significantly to above 5000 nm indicating an unstable emulsion above 50 mM NaCl. This was supported by extensive aggregation with the formation of some very large non-spherical flocs as observed in the optical micrographs (Figure 3A).

The zeta-potential of the emulsion differed between the pH values but did not change significantly with different concentrations of NaCl *($p > 0.05$)*. The zeta-potential of the emulsion at pH 6 and 8 remained below -40 mV and were stable to droplet aggregation. However, in case of the emulsions at pH 2, the ζ-potential of the emulsions exhibited an appreciable decrease from a net positive charge (+17 mV) to approximately 0 mV as the ionic strength was increased to 150 mM NaCl. The ζ-potential of the droplets at pH 2 and 4 were low even in the absence of added salt (Figure 2), so the alternation in ionic strength had no significant effect on the intrinsically unstable emulsions.

As expected, the flocculation of emulsion droplets by added salt at both pHs 2 and 4 was evidenced by extensive creaming (CI_C > 50%, CI_C > 30%) with two distinct layers of cream and serum observed after seven days of storage at room temperature (Figure 3C). On the other hand, the emulsion samples in presence of 250 mM NaCl at pHs 6 and 8 showed no evidence of creaming instability since the cream indices of the serum was zero and cream layer was less than 8% even after 7 days of storage. This indicated that the emulsions were relatively stable to mono valent ion-induced aggregation, which might be attributed to the fact that globulin, a major fraction of the extracted TSP is salt soluble (Liadakis, et al., 1995). The TSP showed 93.4% solubility in NaCl.

Effect of $CaCl_2$ on the properties of TSP-stabilized emulsions

As a part of nutritional improvements, food industries tend to voluntarily fortify food and beverages with different micronutrients, calcium being the most common one (García, Morales, & Sánchez, 2011) It is therefore important to establish the effects of $CaCl_2$

342 composition on the stability and physicochemical aspects of TSP-stabilized emulsions. In this
343 study, four emulsions were prepared (pH 2, 4, 6 and 8) with increasing concentration of
344 $CaCl_2$ (25mM to 250mM). As shown in Figure 4 A, the mean diameter increased
345 dramatically for all emulsions above 100 mM $CaCl_2$ concentration irrespective of pH. For
346 instance, at pH 8, the droplet size was 863 nm in presence of 25 mM $CaCl_2$ and increased
347 significantly to 5300 nm at 250 mM $CaCl_2$. At pH 2-6, addition of 250 mM $CaCl_2$ resulted in
348 appreciable increase of droplet size above 4000 nm and generally the droplet size continued
349 to increase with increasing concentration of $CaCl_2$. The results shown indicate that the TSP-
350 stabilized emulsion was strongly affected in presence of $CaCl_2$ in contrast to NaCl. When the
351 emulsions treated with $CaCl_2$ were mixed gently with 2 wt% SDS solution (sodium dodecyl
352 sulphate) (data not shown), the hydrodynamic diameter reverted back to the size similar to
353 that of the original emulsions with no added salts. This suggests that the increase in droplet
354 diameter was possibly due to ion-induced aggregation rather than droplet coalescence
355 (Keowmaneechai & McClements, 2002).

356 The hydrodynamic diameter results in Figure 4A corroborated well with decrease in
357 the absolute zeta-potential values (Figure 4B) of all emulsion tending to below –20 mV with
358 an increase in $CaCl_2$ concentration. The ζ-potential values of the emulsions decreased from a
359 net positive charge (+12 mV) to approximately 0 as the salt concentration was increased to
360 100 mM (Figure 4B). The significant decrease in ζ-potential of the droplets in presence of
361 $CaCl_2$ might have occurred due to either screening the electrostatic charges by reducing the
362 Debye screening length and/or ion binding of the added chloride ions to the amino groups on
363 TSP (Tangsuphoom & Coupland, 2008; Walstra, 1986). The relative significance of the two
364 phenomena can be examined using colloidal principles. The ζ-potential of a charged surface
365 is related to its surface charged density (σ) by the following equation (Hunter, 2001):
366

$$\sigma = \varepsilon_O \varepsilon_R \kappa \zeta \quad (1)$$

where, ε_O is the dielectric constant of vacuum, ε_R is the relative dielectric constant of the aqueous surrounding medium, and κ^{-1} is the Debye screening length (Walstra, 1986):

$$\kappa = 3.2 \times 10^9 \sqrt{I} \quad (2)$$

where, I is the ionic strength of the electrolyte solution surrounding the charged droplets

$$I = {}^1/_2 \sum m_i z_i^2 \quad (3)$$

where, m_i and z_i are the molarity and zalency of electrolyte ions of type i. In absence of ion binding, the surface charge density of protein-stabilized emulsion droplets is assumed to be constant. In other words, Eq (1) suggests that ζ-potential should decrease as the electrolyte concentration (inverse of Debye screening length) is increased. The magnitude of this decrease in ζ-potential was predicted (Table 1). The ion concentrations were calculated from the amounts of $CaCl_2$, HCl, and NaN_3 present in the emulsions. It was not possible to reliably calculate the surface charge density in the emulsion containing no $CaCl_2$ because the ionic strength was so low that a small uncertainty in the ion concentration caused a dramatic change in the surface charge density (Kulmyrzaev, Chanamai, & McClements, 2000). The surface charge density of the emulsion droplets were calculated by inserting the overall ionic strength and the measured ζ-potential values in to Eqs (1) and (2). The predicted ζ-potential (shown as ζ*) values with increasing $CaCl_2$ concentration assuming constant σ were significantly less negative as compared to the corresponding experimental values (Table 1). These discrepancies between predicted and observed values clearly suggest that the positively charged Ca^{2+} ions were bound to the COO^- groups on the protein and thus reducing the negative ζ-potential values. It is worth noting that calcium ions are prone to specifically bind with carboxylic acid groups (Chakrabarti, 1990), so tomato seed protein being rich in

391 glutamic acid might also structurally bind Ca^{2+} ions. This is in line with previous study where
392 the calcium precitatibility was found to be 32% in salt extracted tomato seed protein solution
393 which highlights the binding of Ca2+ ions to the protein irrespective of being present at
394 native or adsorbed state (Liadakis, et al., 1995).

395 As expected from the droplet size data, the addition of $CaCl_2$ to the emulsions
396 significantly altered the extent of droplet aggregation and therefore caused a significant
397 increase in the creaming indices of the droplets in the emulsion. All emulsions, regardless of
398 the concentration of $CaCl_2$ above 100 mM (Figure 4C) displayed a clear phase separation
399 with a sharp boundary between the cream and serum layers at pH values 2 to 8. The optical
400 micrographs (Figure 5A-F) of the TSP stabilized emulsions in presence of increasing $CaCl_2$
401 showed pronounced droplet aggregation and were generally consistent with the data of low ζ-
402 potential values, extensive creaming behavior and large droplet diameter. The extent of
403 aggregation increased as a function of $CaCl_2$ concentration. Interestingly, at 150 mM $CaCl_2$
404 concentration (Figure 5D), a network structure of flocculated droplets was formed, which
405 appeared to be more noticeable at higher $CaCl_2$ concentration ((Figure 5E-F). However, no
406 coalescence was observed even at 250 mM $CaCl_2$ (Figure 5F) as discussed previously, which
407 might be attributed to the steric repulsive (or distance barrier) of larger protein particles that
408 have already been adsorbed. The formation of aggregates appeared to cause the flocs to rise
409 under gravity to the top of the emulsion leaving behind a clear serum (Figure 4C). However,
410 there was no increase in creaming indices in spite of increase of droplet diameter above 100
411 mM $CaCl_2$ (data not shown) , which might be attributed to the network structure of
412 flocculated droplets which remained connected, and extended throughout the container
413 volume, as previously observed in whey protein concentrate-stabilized systems (Ye & Singh,
414 2000). These results highlight that multivalent counter-ions such as Ca^{2+} might be more
415 effective in promoting emulsions instability in TSP-stabilized emulsions as compared to

monovalent counter-ions such as Na^+ due to charge screening effects and ion binding effects in case of the $CaCl_2$ systems (Kulmyrzaev, et al., 2000).

Effect of temperature on the properties of TSP-stabilized emulsions

To prevent food borne illnesses and microbial spoilage, thermal treatments such as pasteurization, sterilization and cooking are commonly used in food industries. Such temperature conditions can influence the stability of commercial products containing protein-coated lipid droplets. Consequently, it is important to investigate the influence of heating on the stability and physicochemical properties of TSP-stabilized emulsion. The influence of holding temperature was thus examined over a range of 30-90°C, with a holding time for 30 minutes. As it can be observed in Figure 6, heat treatment of the emulsions at 30-70 °C had hardly any influence on the mean droplet diameter and ζ-potential ($p > 0.05$). However, the droplet size increased significantly to 4767 nm and 9560 nm, when subjected to heat treatment at 80 °C and 90°C, respectively. The particle size measured by dynamic light scattering was in agreement with optical micrograph showing densely packed aggregates of oil droplets (Figures 5A and B). As expected, the TSP-coated droplets were relatively insensitive to thermal processing in the temperature range of 30-70°C with ζ-potential remaining at ~-40 mV. However, there was a pronounced decrease in magnitude of electrical charge ≥80 °C to -22 mV.

The increase in droplet size could point towards the occurrence of heat-induced droplet aggregation. A possible explanation for the aggregation is that thermal denaturation and subsequent conformational changes of the adsorbed globular proteins could have occurred at 80 °C (Wang, et al., 2012). Tomato seeds contains high level of the globulin fraction (14.0%) followed by a glutelin fraction (4.0%), albumins (2.6%), and prolamines (1.2%) (Piyakina, Maksimov, & Yunusov, 1998). Consequently, the exposure of the reactive

groups originally located within the TSP globular proteins due to heat treatment might causes an increase in surface hydrophobicity of the protein-coated droplets. Since not all hydrophobic side chains might not be oriented towards the oil phase, such increase in hydrophobic moieties can generate protein-protein interaction between protein-coated droplets, which might result in aggregation. Extensive droplet aggregation observed at the heating temperatures of 80-90°C indicates that the denaturation temperature of TSP such might be in that region. These results are in agreement with previous reports (Savadkoohi & Farahnaky, 2012), where differential scanning calorimetry thermal curves of TSP solution showed that the denaturation temperature of the globular fractions of TSP in 1-3 wt% TSP solution was at 86-87 °C.

Conclusions

This study examined the influence of environmental stresses, such as pH, NaCl, $CaCl_2$ and heat treatment on the stability of TSP-stabilized oil-in-water emulsions. It was shown that TSP-stabilized emulsion droplets were prone to droplet flocculation and creaming at pH values close to their isoelectric point (pH 2-4). The stability of the emulsions to salt addition depended on the pH and valency of ions, with emulsions at pH 6-8 being relatively stable to high levels of NaCl upto 250 mM NaCl. In contrast, the TSP-stabilized emulsions were unstable at all pH values after the addition of $CaCl_2$ (25 – 250 mM $CaCl_2$). The emulsion stability did not alter markedly after heat treatment at 30°C- 70°C. Thus, TSP appears to retain its emulsifying properties even after pasteurization. However, ≥80°C, there was an extensive droplet aggregation which was attributed to the denaturation of the TSP globular protein fractions. Under no conditions, emulsions showed any droplet coalescence during the period of storage. Therefore, from this study, it can be inferred that TSP has the potential to become an emulsifier with good emulsion stabilizing properties for use in neutral food

applications. The use of TSP as an emulsifier would not only reduce the amount of waste product produced and subsequent landfill issues but also generate an interesting low-cost plant protein based novel emulsifier.

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586

587

588 **Table 1.**

$CaCl_2$ (mM)	ζ-potential (mV)	σ ($\times 10^{-2}$ Cm^{-2})	ζ^*-potential (mV)
25	-31	-2.92	-31
50	-22	-4.12	-33
100	-17	-5.83	-24
150	-15	-7.14	-19
200	-14	-8.25	-17
250	-11	-9.22	-15

589

590

Figure 1.

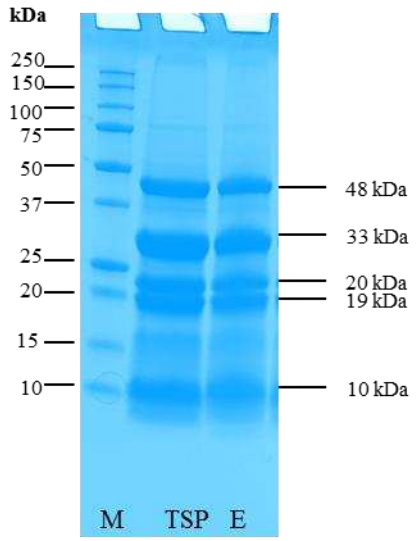

kDa
250
150
100
75
50
37
25
20
15
10
48 kDa
33 kDa
20 kDa
19 kDa
10 kDa
M
TSP
E

Figure 2.

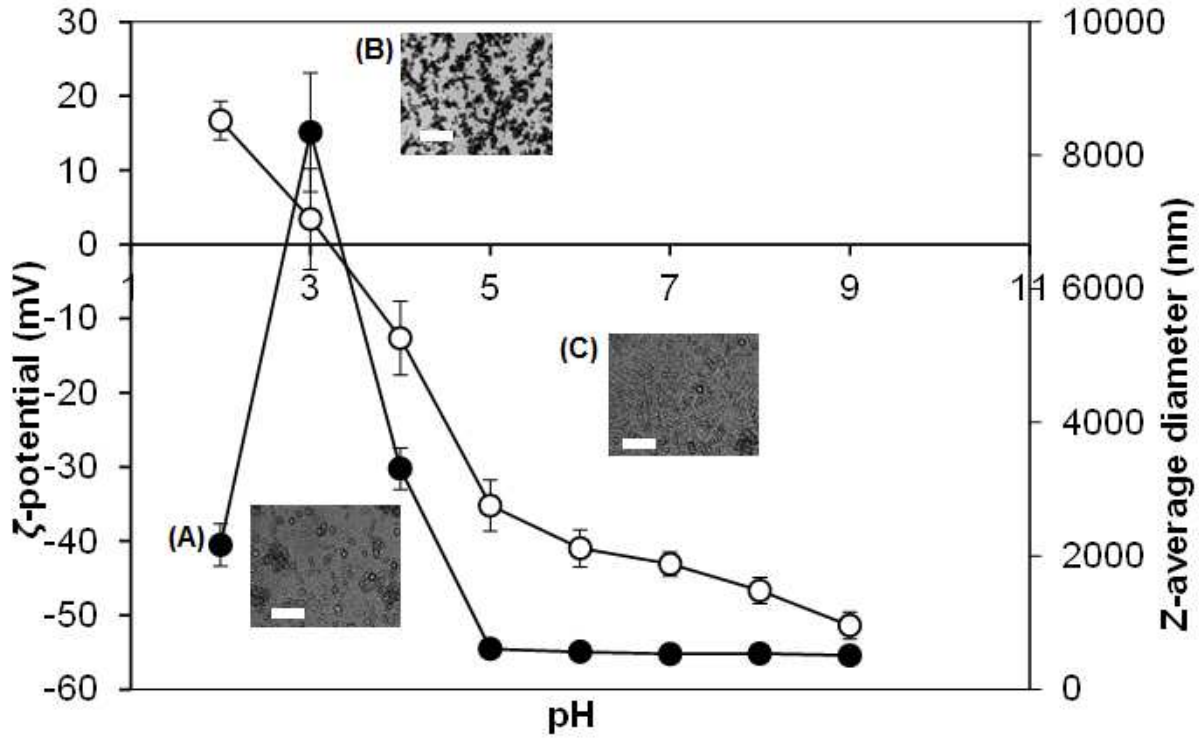
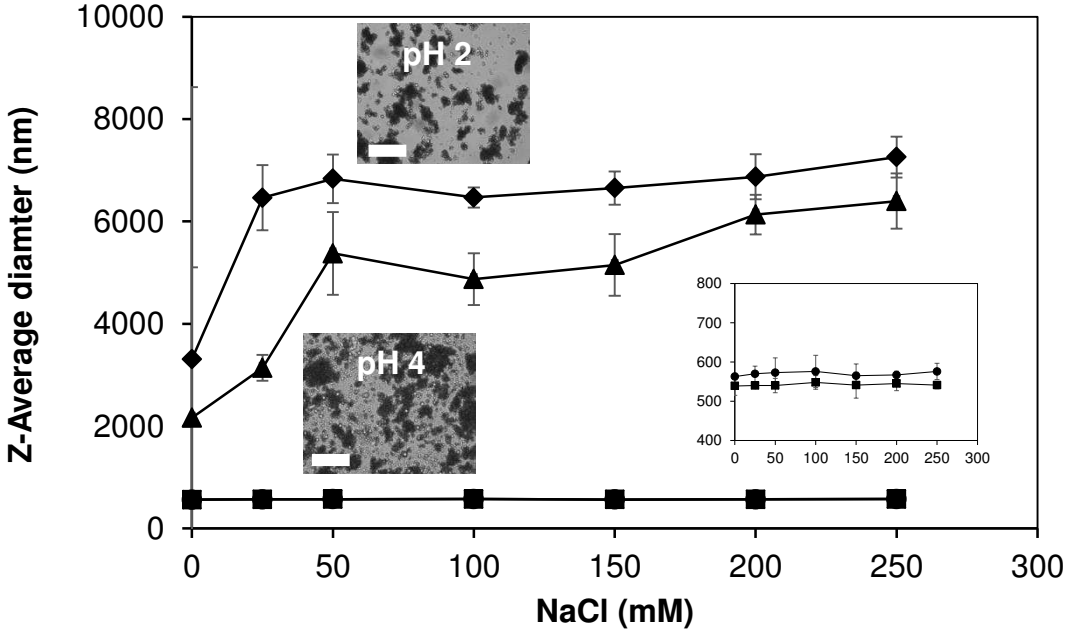
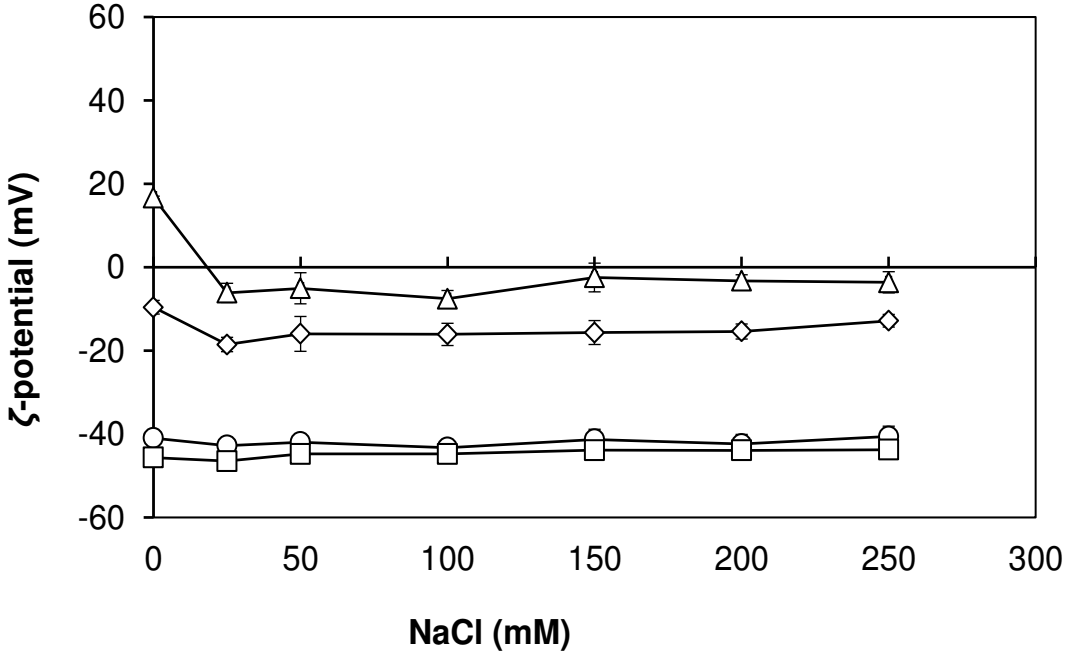

30
20
10
0
-10
-20
-30
-40
-50
-60
ζ-potential (mV)
3
5
7
9
11
pH
10000
8000
6000
4000
2000
0
Z-average diameter (nm)
(A)
(B)
(C)

Figure 3.

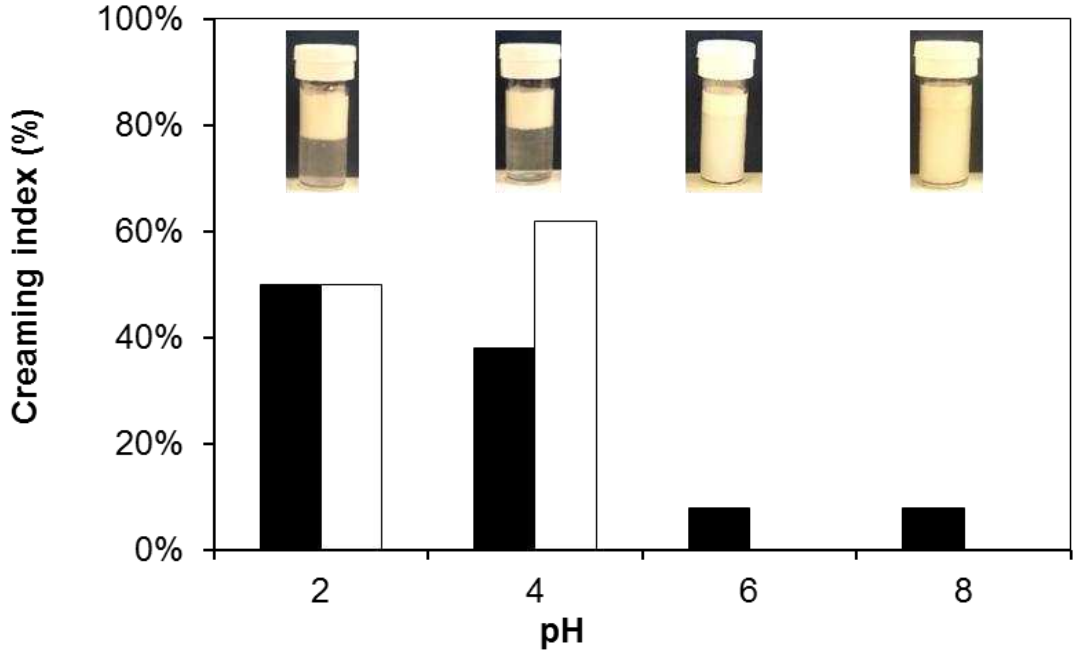
(A)


Z-Average diamter (nm)
10000
8000
6000
4000
2000
0
0
50
100
150
200
250
300
NaCl (mM)
pH 2
pH 4
800
700
600
500
400
0
50
100
150
200
250
300

(B)


ζ-potential (mV)
60
40
20
0
-20
-40
-60
0
50
100
150
200
250
300
NaCl (mM)

600 **(C)**



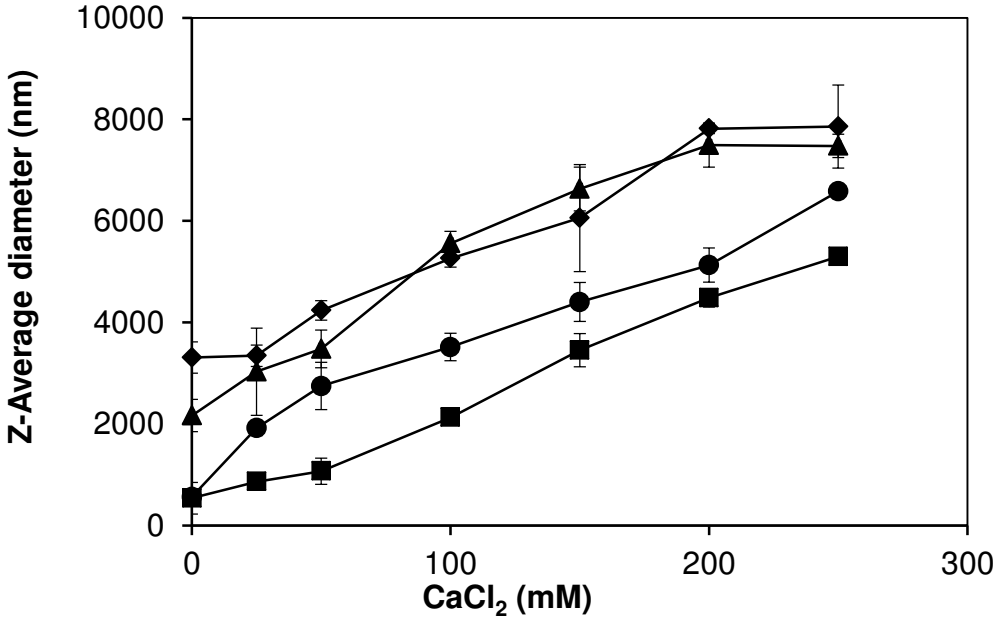
601

602

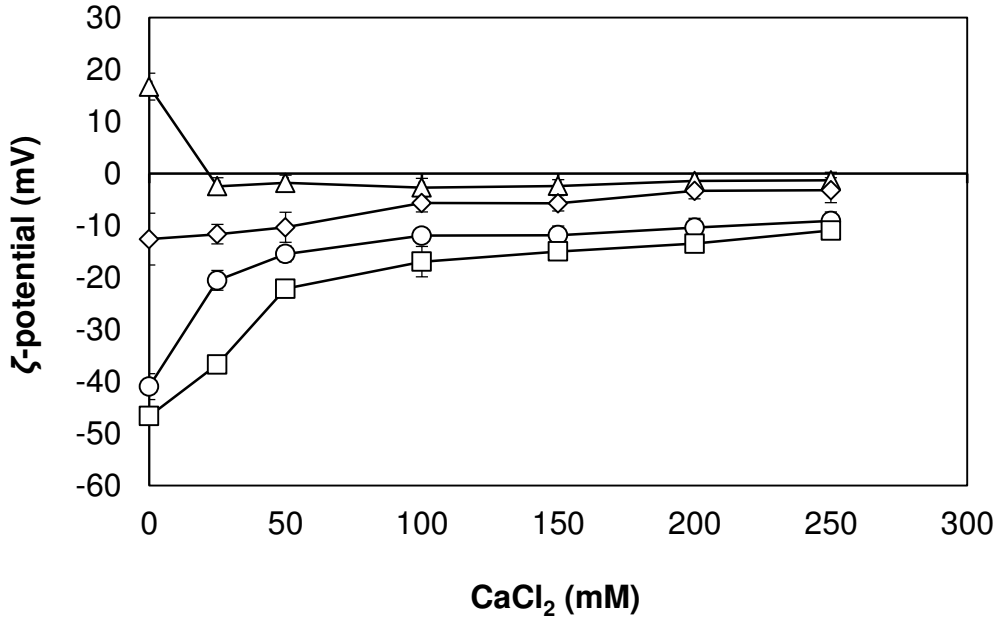
603

604 **Figure 4.**

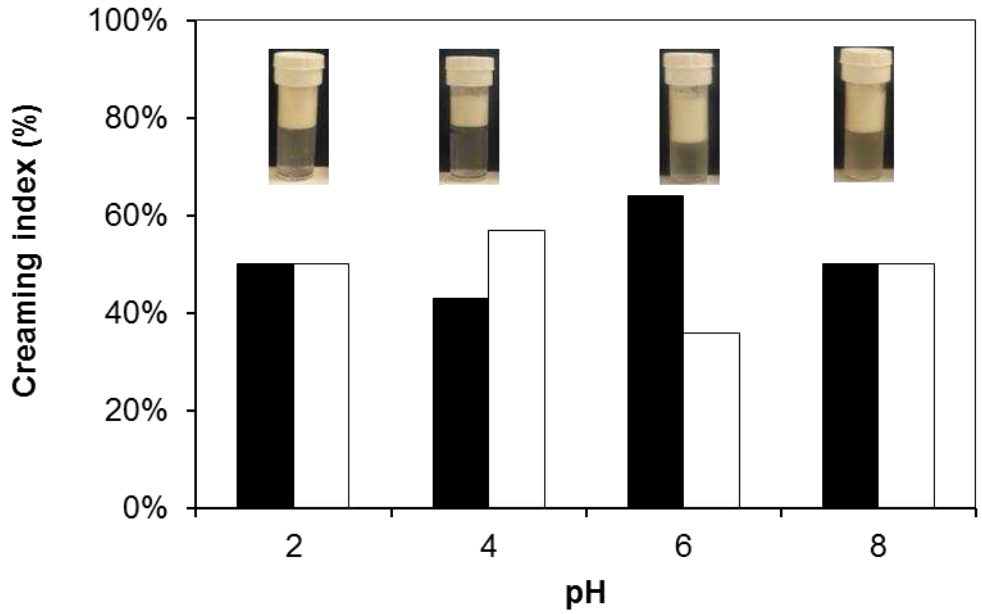
(A)



(B)

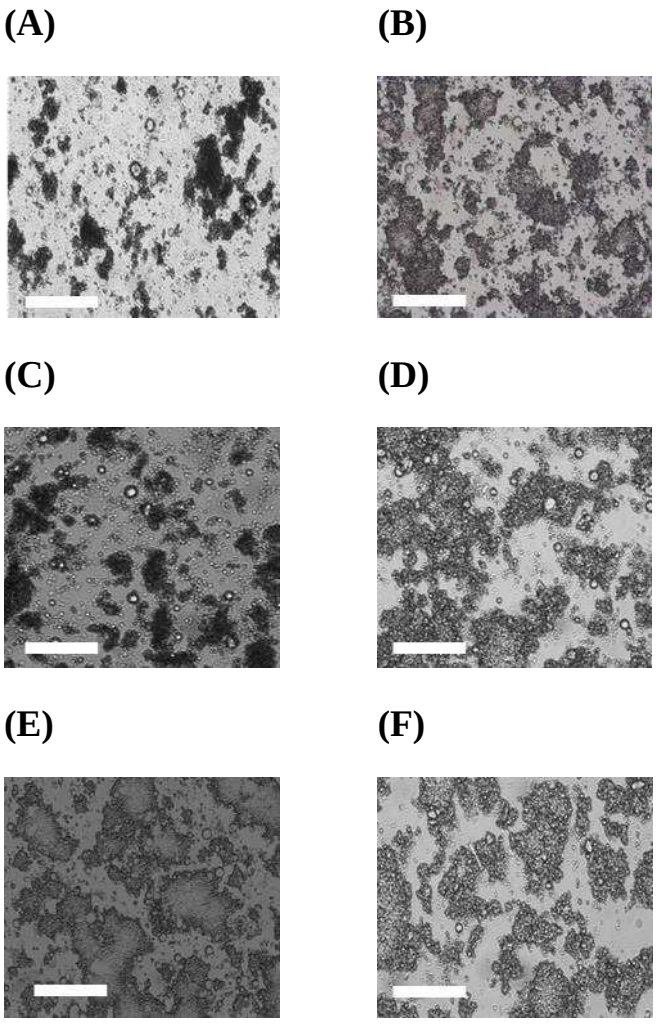


(C)



605

606 **Figure 5.**



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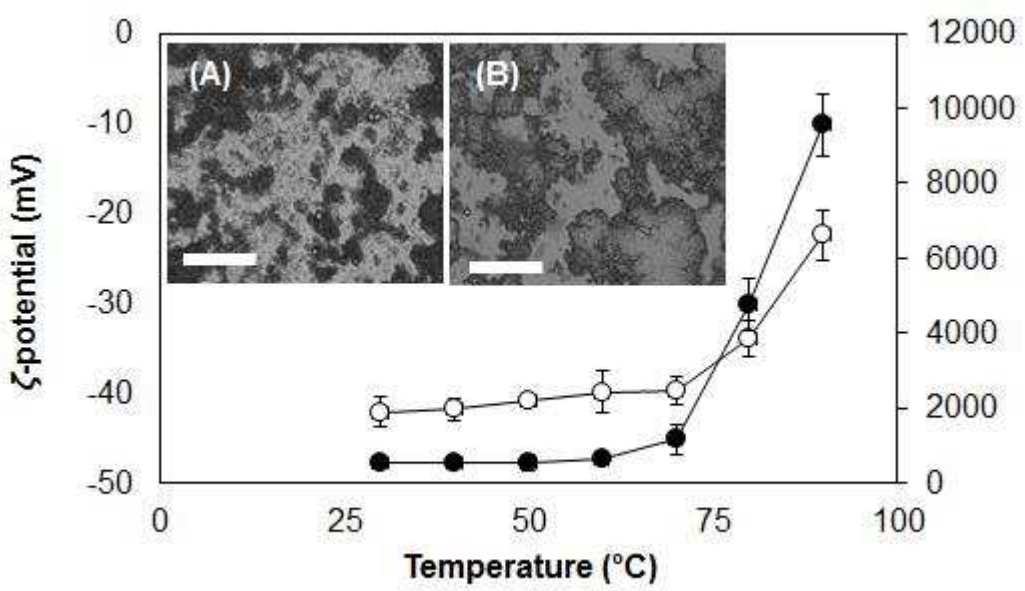
608

609 **Figure 6.**



610