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Research Article

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Eggshell waste transformation to calcium chloride anhydride as food-grade additive and eggshell membranes as enzyme immobilization carrier

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Abstract: In continuation of our efforts to fully utilize eggshell waste (ESW), here we report the possibility of ESW transformation to calcium chloride (CaCl_2) anhydride of food-grade additive purity and eggshell membranes (ESMs) as potential enzyme immobilization carriers. ESW chemical transformation by 5% (w/v) hydrochloric acid to CaCl_2 solution and ESM completely devoid of the remnants of ESW calcium carbonate was performed in the constructed 15 L batch reactor during 4 h at room temperature, followed by separation of ESM from CaCl_2 solution by filtration. ESW-derived CaCl_2 solution containing the excess hydrochloric acid was neutralized by adding calcium hydroxide, concentrated to approximately 1/8th of volume, and spray dried. Separated ESM was washed with water and acetone, dried, and ground to a size of less than 0.5 mm. The ESW transformation process produced 102.42 ± 3.31 g of CaCl_2 anhydrous and 2.48 ± 0.28 g of ESM per 100 g of ESW dry matter. ESW-derived CaCl_2 fulfilled all criteria for food-grade additive,

while obtained ESM showed their suitability for *Burkholderia cepacia* lipase immobilization by adsorption.

Keywords: eggshell waste utilization, hydrochloric acid treatment, calcium chloride anhydride, eggshell membranes, lipase immobilization

1 Introduction

Calcium chloride (CaCl_2) is an inorganic salt of calcium and chlorine, registered in the European Union (EU) under the Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), whose manufacturing and/or import to the European Economic Area comprise about 100,000 tons annually [1]. It has been used in road deicing, dust control, road stabilization, concrete curing, oil well drilling, tire ballasting, for the production of washing and cleaning products, anti-freeze products, fertilizers, plant protection products, adsorbents, water treatment chemicals, and heat transfer fluids, as well as food-grade additive [2–5].

Currently, the industrial production of CaCl_2 is based on three different processes: (a) hydrochloric acid treatment of limestone as the dominant one, where CaCl_2 is produced by chemical reaction between limestone calcium carbonate and hydrochloric acid; (b) Solvay process where the reaction between sodium chloride and calcium carbonate in the presence of ammonia as the catalyst results with sodium carbonate as the main product and CaCl_2 as a by-product of the chemical reaction; and by (c) purification of naturally occurring brine CaCl_2 (such as those one present in salt lakes) [2–5].

Besides limestone, which is a natural and geologically created deposit of calcium carbonate in the form of calcite crystals, another natural form of calcite crystal is the eggshell [6], accounting for the major part of eggshell waste

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(ESW) produced by households, restaurants, and various industries [7–9]. Based on the latest available report of the Food Agricultural Organization (FAO) on hen egg production for the year 2021 [10], and the most conservative estimate that ESW accounts for 10% of total egg mass [8,9], it is estimated that about 8,638,779 tons of ESW are generated worldwide, with 1,111,751 tons in the EU. Moreover, considering the fact that about 30% of totally generated ESW in the industrialized countries are the by-product of egg-breaking plants [11], it can be estimated that about 2,591,634 tons of worldwide generated ESW and about 333,525 tons of ESW in the EU has been generated by egg-breaking plants. Since the disposal of ESW generated by egg-breaking plants is quite costly [7,8,11], there is an obvious need to reduce such costs by implementing at least some of the strategies for ESW transformation. These include ESW transformation to the low-value-added products such as fertilizers or feed for animals and pets [7,12,13], or implementation of novel strategies of sustainable waste management oriented toward *zero-waste* model, where innovative waste transformation techniques for the production of chemicals, fine chemicals, bioactive compounds, enzymes, and functional materials have been applied [7–9,14].

Besides well-defined possibilities of ESW transformation to the value-added products reported in Cordeiro and Hincke [15], Waheed *et al.* [16], and Ahmed *et al.* [11], we would like to point out two recently published articles of our research group, where the possibility of the complete utilization of ESW collected from household and restaurants by chemical treatment with 5% (w/v) hydrochloric, 10% acetic (w/v), and 15% *o*-phosphoric acid (w/v) to the corresponding calcium salts and ESW-derived eggshell membranes (ESMs) were performed on a laboratory scale [8,9]. Therefore, it seems quite possible to extend current strategies of the ESW transformation process to the novel ones, more oriented toward *zero-waste* model of the complete ESW utilization.

In this regard, we have examined the possibility of ESW transformation by 5% (w/v) hydrochloric acid treatment upscaling in the constructed 15 L conical batch reactor, followed by the subsequent production of CaCl₂ anhydride by spray drying, and ESM-derived ESMs as potential enzyme immobilization carrier. ESW-derived CaCl₂ anhydride was tested on its suitability as food-grade additive, while eggshell-derived ESM was tested on the key characteristics as a potential enzyme immobilization carrier, as well as its suitability to be used as an enzyme carrier for *Burkholderia cepacia* lipase (BCL) immobilization by adsorption.

2 Materials and methods

2.1 Materials

Industrial ESW was generously supplied by Elcon-Nutritional Products Ltd. (Zlatar Bistrica, Croatia).

The transformation process of ESW was performed by 5% (w/v) HCl prepared from hydrochloric acid (37% w/v) purchased from Carlo Erba (Emmendingen, Germany), while the excess of hydrochloric acid in the obtained CaCl₂ solution was neutralized by adding calcium hydroxide purchased from Across Organics (Geel, Belgium). Complexometric determination of calcium content was performed using calconcarboxylic acid obtained from Acros Organics (Geel, Belgium) and the disodium salt of ethylenediaminetetraacetic acid from Fischer Scientific (Leicestershire, UK).

Amano lipase PS from *Burkholderia cepacia* (Sigma–Aldrich product number: 534641) used for immobilization on the obtained ESMs was purchased from Sigma–Aldrich Chemicals (Saint Louis, MO, USA).

All other chemicals used in this research were of *pro-analysis* purity.

2.2 Chemical analysis of ESW

Chemical analysis of originally supplied ESW, as well as those prepared by tap water washing 3 × 10 min of originally supplied ESW, included determination of dry matter, total protein, lipid, and calcium carbonate content, all performed according to Strelec *et al.* [8].

2.3 ESW transformation process

The ESW transformation process using 5% (w/v) hydrochloric acid was modeled based on the previously reported ESW transformation with 5% HCl [8], but with some modifications. These included: (a) process scaling up (~7.2-fold), (b) using 15 L conical batch reactor instead of glass beakers, (c) ESW washing with tap water instead of distilled one, (d) replacing calcium salt precipitation by acetone with spray-drying of concentrated solution, and (e) omitting the grinding step of obtained dried CaCl₂.

The transformation process (Figure 1) started with 3 × 10 min washing of 650 g of ESW with 6.5 L of tap water in a constructed 15 L conical batch reactor equipped with an

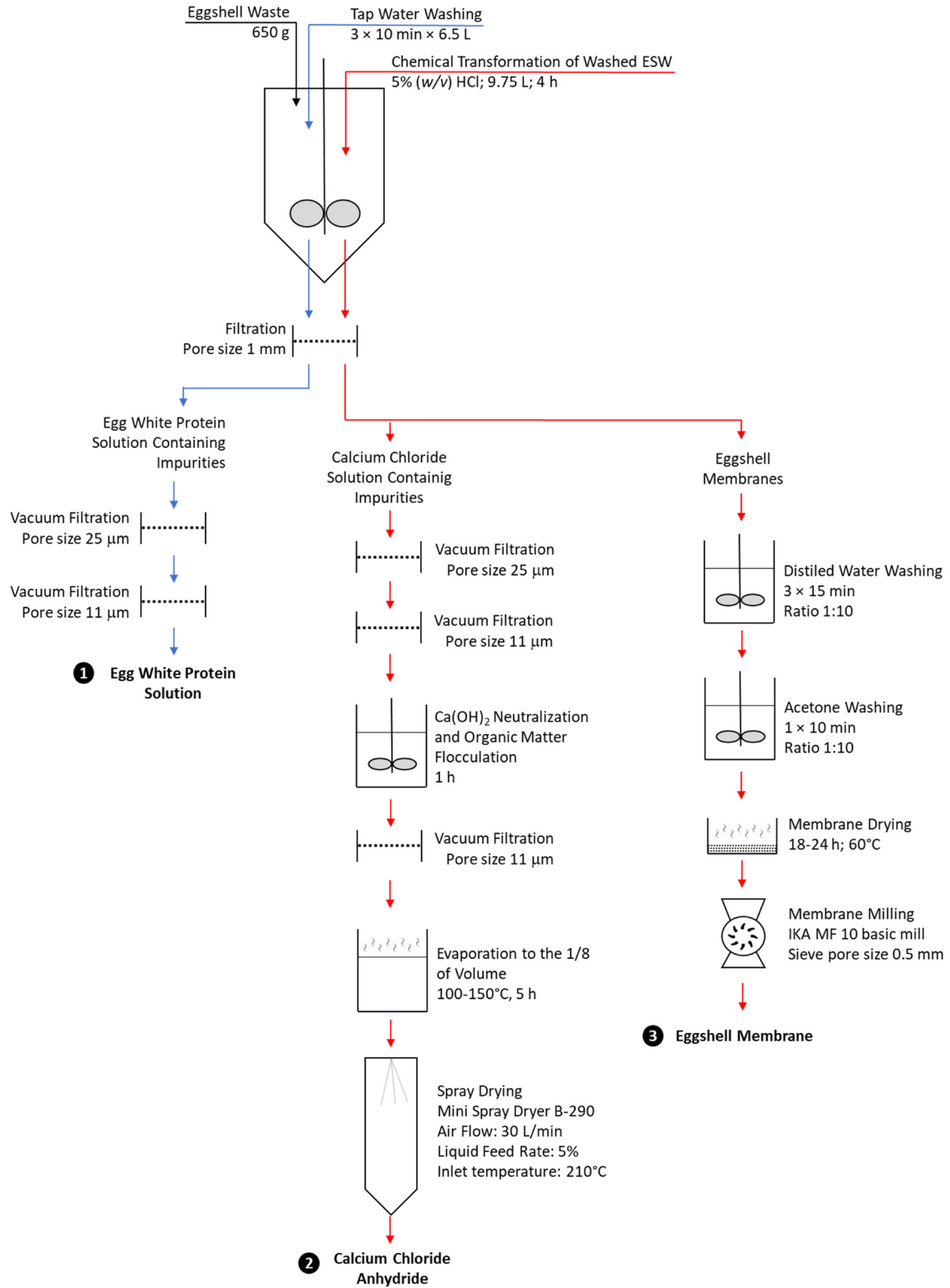


Figure 1: Schematic presentation of transformation process of industrial eggshell waste to egg white protein solution, calcium chloride anhydride, and eggshell membranes.

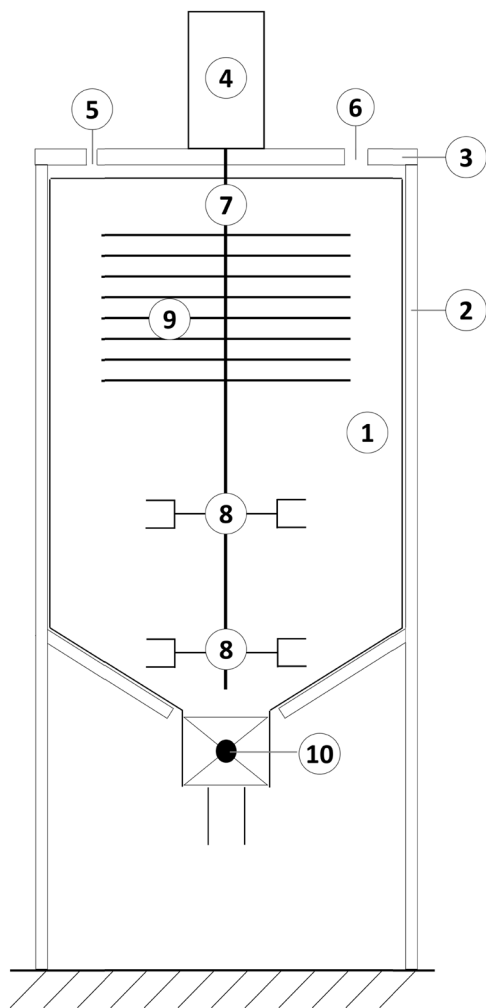


Figure 2: Schematic presentation of the constructed 15 L conical batch reactor for eggshell waste transformation. 1 – polyethylene terephthalate 15 L batch reactor ($\phi = 240$ mm; $h = 380$ mm); 2 – conical batch reactor holder; 3 – poly(methyl methacrylate) overhead stirrer holder; 4 – variable speed overhead stirrer with the possibility of contrarotation; 5 – sampling opening; 6 – acid dosage opening; 7 – acid resistant stainless steel rotating shaft; 8 – acid resistant stainless steel double impulse stirring elements ($\phi = 110$ mm); 9 – acid resistant stainless steel comb type stirring element (foam breakers; $\phi = 170$ mm, $h = 100$ mm, 8 wires); and 10 – polyvinyl chloride un-plasticized (PVC-U) ball valve type 546 Pro.

overhead variable speed stirrer set at $350 \text{ rpm}\cdot\text{min}^{-1}$, and the use of two double impulse type stirring elements set at 7 and 15 cm from the bottom of the reactor (Figure 2).

Each of the protein solutions obtained after ESW washing was successively filtered through filter paper Whatman 114 followed by Whatman 1. The protein content of the clear protein solutions was determined using the Bradford technique [17].

The ESW obtained after three steps of washing was mixed with 9.75 L of 5% (w/v) hydrochloric acid in a 15 L batch reactor, but the addition of HCl was performed in

aliquots as follows: 1 L of HCl was added to ESW at the beginning, followed by addition of 0.5 L each 10 min, and 0.75 L in the last step.

Mixing conditions of ESW suspension in HCl were performed at two different speed rates, as follows: 5% (w/v) HCl aliquot was added to washed ESW and mixed at $150 \text{ rpm}\cdot\text{min}^{-1}$ for 5 min in order to prevent excessive foaming; afterward, the mixing speed was then increased to $250 \text{ rpm}\cdot\text{min}^{-1}$. In both cases, excessive foaming was additionally reduced by the use of comb-type stirring elements. The process of the complete transformation of ESW calcium carbonate to CaCl_2 by HCl and subsequent release of ESM completely devoid of the remnants of ESW- CaCO_3 was finished at the fourth hour. Afterward, ESM was separated from the CaCl_2 solution by filtration using a plastic mesh screen with a pore size of 1 mm.

The obtained ESM was washed with distilled water and acetone, dried, and milled to less than 0.5 mm, according to Strelec *et al.* [8].

The ESW-derived CaCl_2 solution obtained after separation from ESM was subjected to two successive steps of vacuum filtration through filter paper Whatman 114 and then through Whatman 1. A clear solution of ESW-derived CaCl_2 was then subjected to the neutralization of the excess of HCl by calcium hydroxide addition, followed by organic matter flocculation, clarification by vacuum filtration, and evaporation to the 1/8th of volume as previously reported by Strelec *et al.* [8].

The concentrated solution of ESW-derived CaCl_2 was cooled to room temperature and then spray-dried with Mini Spray Dryer B-290 (Büchi, Postfach, Switzerland) at determined optimal conditions: an inlet temperature of 210°C , an airflow on nozzle of $30 \text{ L}\cdot\text{min}^{-1}$, an aspirator rate of 100% (equal to flow of $35 \text{ m}^3\cdot\text{h}^{-1}$ of drying air), and a liquid feed rate of $1.5 \text{ mL}\cdot\text{min}^{-1}$. In addition, the determination of optimal conditions for spray drying of ESW-derived CaCl_2 included variations in liquid feed rate between 1.5 and $6.0 \text{ mL}\cdot\text{min}^{-1}$, and airflow between 20 and $30 \text{ L}\cdot\text{min}^{-1}$.

CaCl_2 production was measured by determining CaCl_2 , protein, and amino sugar content in the solution. Calcium content was determined by complexometric titration [8], protein content by the Bradford method [17], and amino sugar content by the Morgan–Elson method [18].

2.4 Characterization of ESW-derived CaCl_2 anhydride

ESW-derived CaCl_2 powder was spray-dried and examined for chemical composition, suitability as a food-grade

additive, XRD pattern, and microbiological purity. Dry matter, CaCl_2 and soluble protein content, CaCl_2 powder solubility in water, pH of 10% CaCl_2 powder solution, and free alkali content were determined according to Strelec et al. [8]. The total protein content in the obtained CaCl_2 powder was determined by the standard Kjeldahl method, while the amino sugar content in CaCl_2 solution of $100 \text{ mg}\cdot\text{mL}^{-1}$ by the Morgan–Elson method [18]. The Andrija Štampar Teaching Institute of Public Health (Zagreb, Croatia) assessed Mg and alkali salt content in CaCl_2 powder using the gravimetric methods, fluoride content using an ion selective method, and Mg, F, As, Pb, and Hg using the ICP-MS technique.

FTIR-ATR analysis of CaCl_2 powders produced on Mini Spray Dryer B-290 was performed on a Carry 630 FTIR ATR spectrometer (Agilent, Santa Clara, CA, USA) in the range of $650\text{--}4,000 \text{ cm}^{-1}$.

Powder X-ray diffraction measurements of ESW-derived CaCl_2 powders were performed using a Bruker Discover D8 diffractometer (Karlsruhe, Germany) supplied with a LYNXEYE XE-T detector in the angular 2θ range $10\text{--}70^\circ$ with a step size of 0.02° and a measuring time of 1 s per step. The Eva software was used to identify the crystal phases, followed by a search and match method in the PDF4 database. Rietveld structural refinement was performed using the FULLPROF software [19].

Microbiological quality criteria of ESW-derived CaCl_2 powders included determination of total aerobic plate count, mesophilic and thermophilic spore count, yeasts, molds, as well as pathogenic microorganisms including fecal coliforms and *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*, all performed by standardized microbiological procedures [20–24].

2.5 Examination of ESW-derived ESMs as potential enzyme immobilization carrier

ESW-derived ESMs were evaluated for their suitability as potential enzyme immobilization carriers. This included the determination of particle size distribution, water and oil holding capacity (WHC and OHC), as well as the possibility of immobilization of BCL onto the produced ESM by adsorption.

Particle size distribution of the produced ESM grounded to a size less than 0.5 mm was investigated by the laser light scattering method using a Mastersizer Scirocco 2000 analyzer (Malvern Instruments, Malvern, UK). The obtained results are presented as two dependent parameters: volume weighted mean diameter and span value, which were averaged values from three measurements.

WHC and OHC of the obtained ESM was performed by the method from Ballesteros et al. [25] with minor modifications. In brief, 0.5 g of the ESM sample was mixed with 5 mL of distilled water or olive oil, vortexed for 1 min, and centrifuged at $2,795 \times g$ during 30 min in a Centric 150 centrifuge (Tehtnica, Podplat, Slovenia). Afterward, the volume of supernatant was measured, with WHC represented as mL of water per gram of the ESM sample and OHC as mL of olive oil held per gram of the ESM sample.

BCL immobilization by adsorption on the produced ESM was performed by the combination of methods of Chattopadhyay and Sen [26] and Salleh et al. [27], during 1, 2, 3, 4, 5, and 6 h with constant 360° stirring/rotation of the BCL and ESM suspension (0.5 g of ESM mixed with 10 mL of BCL of desired total activity) in 15 mL Falcon tubes on Multi-Rotator PTR-60 (Grant-Instruments Ltd, Cambridgeshire, UK) set at $17 \text{ rpm}\cdot\text{min}^{-1}$, at room temperature. BCL solutions in 50 mM phosphate buffer at pH 7.5 (10 mL) exhibited desirable total lipase activity of 230, 450, 820, 1,190, and 1,430 U. The effect of time and BCL concentration on immobilization was monitored by titrimetric determination of lipase activity [28], where the activity of immobilized BCL was expressed in units per gram of the ESM carrier ($\text{U}\cdot\text{g}^{-1}$).

3 Results

Encouraged by our previous research on the possibility of ESW transformation, on a laboratory scale, to the egg white protein solution, CaCl_2 dihydrate, and ESM powder of ESW collected from households and restaurants [8], here we report the possibility of transformation of “industrial” ESW collected from egg-breaking plant to CaCl_2 anhydride as a food-grade additive, and ESMs as a promising enzyme immobilization carrier. Although the currently proposed ESW transformation procedure (Figure 1) included the majority of previously reported transformation steps [8], it should be pointed out that the novelty of the proposed ESW transformation lies in scaling-up (7.2-fold) where the glass beaker was replaced by a 15 L conical batch reactor (Figure 2) followed by subsequent changes in stirring conditions, and in the production of CaCl_2 anhydride by spray drying. Therefore, it could be safely concluded that the currently proposed ESW transformation process does not represent a simple scale-up procedure, but a novel and upgraded version of the possibility of ESW transformation to the high-value added products oriented toward a *zero waste* model. Moreover, it seems that the currently proposed ESW transformation procedure in a 15 L conical

batch reactor might present a true intermediate step for upscaling from the laboratory to industrial-scale production.

3.1 Chemical composition of industrial ESW

It is well established that hen egg and eggshell composition is dependent on several factors including genetics and hen breed, the mode of feeding, physiological status (hen-age, stress, and immune status), as well as eggshell color: brown or white [16,29–32]. Therefore, it was necessary to determine the basic chemical composition of the “industrial” ESW regardless of the existing data on ESW composition reported in the literature [8,16,30,33]. This was done in order to calculate the chemical yield of CaCl_2 produced by 5% (w/v) hydrochloric acid ESW treatment and to evaluate the successes of the proposed ESW transformation process.

Table 1 shows the basic chemical composition of originally supplied ESW (unwashed eggshells) and ESW devoid of adherent egg white proteins (washed eggshells) collected from the egg-breaking plant. The major component of both examined ESWs was calcium carbonate (90–92%), while proteins were present in amounts lower than 5.5% and lipids lower than 0.6%. Originally supplied ESW washing with tap water resulted in a slight increase of CaCO_3 in washed and dried ESW and a decrease in the protein content (Table 1), which was congruent with reports of Walton *et al.* [33] and Strelec *et al.* [8], and could be attributed to the removal of a thin layer of adherent egg white present in ESW.

The obtained data on the basic chemical composition of examined ESWs were in agreement with findings by Strelec *et al.* [8], Waheed *et al.* [16], Ray *et al.* [30], and Walton *et al.* [33].

Table 1: Chemical composition of industrial eggshell waste¹

| Content | Unwashed eggshells ² | Washed eggshells ³ |
|--|---------------------------------|-------------------------------|
| Dry matter ($\text{g}\cdot 100\text{ g}^{-1}$) | 85.97 ± 0.03 | 99.97 ± 0.02 |
| Proteins ($\text{g}\cdot 100\text{ g}_{\text{d.w.b.}}^{-1}$) ⁴ | 5.35 ± 0.14 | 2.42 ± 0.15 |
| Lipids ($\text{g}\cdot 100\text{ g}_{\text{d.w.b.}}^{-1}$) ⁴ | 0.59 ± 0.04 | 0.51 ± 0.05 |
| CaCO_3 ($\text{g}\cdot 100\text{ g}_{\text{d.w.b.}}^{-1}$) ⁴ | 90.39 ± 0.86 | 91.43 ± 0.88 |

¹Results are shown as average value \pm standard deviation of five independent determinations.

²Unwashed eggshells – originally supplied ESW.

³Washed eggshells – originally supplied ESW washed 3×10 min with distilled water and dried at 60°C for 24 h.

⁴d.w.b. – dry weight basis.

3.2 Optimization of ESW transformation process

The first step in the optimization of the ESW transformation process (Figure 1) was to determine the time necessary for adherent egg-white protein removal, followed by the time needed for the complete ESW calcium carbonate dissolution using 5% (w/v) hydrochloric acid.

Trial runs on the “industrial” ESW washing by tap water in a 15 L conical batch reactor revealed that 3×10 min washing was sufficient for the complete removal of the adherent egg white proteins (Figure 3a). It should be pointed out that the selected speed of stirring elements was sufficient to ensure suspension homogeneity. The majority of adherent egg white proteins were extracted during the first washing ($\sim 92.89\%$), while remnants of

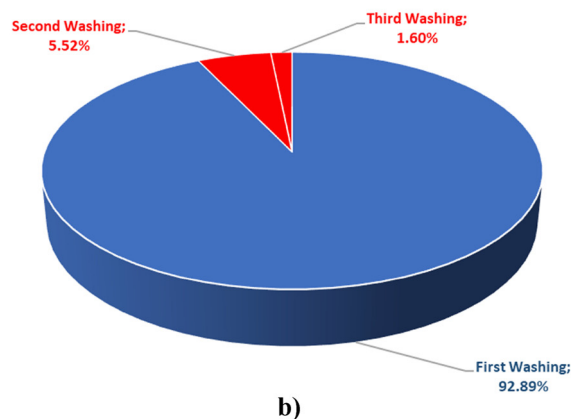
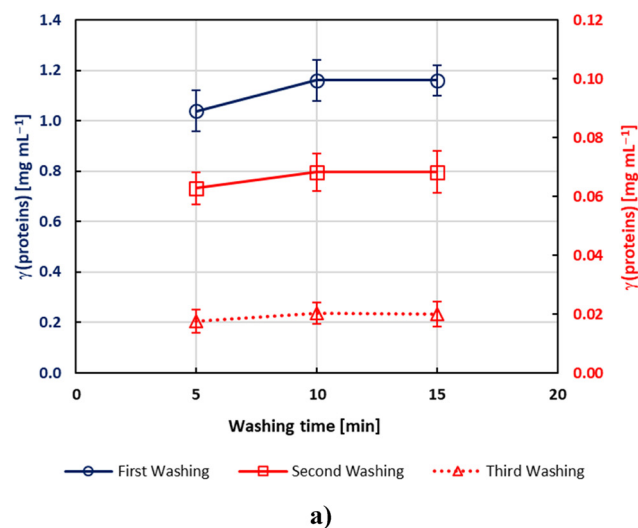


Figure 3: Optimization of industrial eggshell waste washing with tap water in 15 L batch reactor. (a) Mass concentration of proteins present in the solution after first, second, and third washing during 5, 10, and 15 min; (b) overall distribution of washed adherent egg white layer proteins during 10 min washing.

ESW proteins were exhausted during the second (~5.52%) and third (~1.60%) phase of 10 min washing (Figure 3b). Therefore, 3×10 min washing of ESW with tap water was defined as the first step in ESW transformation in a 15 L conical batch reactor.

Obtained data on the percentage of adherent egg white proteins extracted during ESW washing were consistent with our previous report where ESW halves and pieces were washed 3×30 min in glass beaker with distilled water [8]. However, it should be noted that the currently proposed ESW washing time (3×10 min) clearly present improvement from the previous one [8], since ESW washing was threefold reduced (from 90 to 30 min).

Besides the reduction in washing time, “industrial” ESW water washing in a 15 L batch reactor by the use of two double impulse-type stirring elements set at $350 \text{ rpm} \cdot \text{min}^{-1}$ caused breaking of ESW halves and pieces to ESW particles smaller than $4 \text{ cm} \times 4 \text{ cm}$. The reduction in the size of washed ESW had a negative impact on the possibility of addition of the complete volume of 5% (w/v) hydrochloric acid due to uncontrolled foaming and spilling over the edge of the reactor. Therefore, it was necessary to conduct a few trial runs of washed ESW transformation treatment by 5% (w/v) hydrochloric acid, including time-dependent gradual acid dosage in combination with variations in stirring speed.

The combination of temporal acid dosage and changes in variations of stirring speed did not result in the desired foam reduction during the ESW transformation process after two trial runs. Therefore, it was necessary to introduce an additional element, a comb-type stirring element as a foam breaker/reducer (Figure 2). The use of foam breaker/reducer enabled adequate ESW-acid transformation, and after two additional trial runs, a complete protocol of washed ESW transformation by 5% (w/v) hydrochloric acid, defined in sub-Section 2.3, was established. Afterward, three independent “industrial” ESW-transformation batch procedures were performed.

Time-dosage dependent 5% (w/v) hydrochloric acid solvation of washed “industrial” ESW resulted in the prolongation of time from previously reported 3 h [8] to 4 h. Nevertheless, the total time of the ESW transformation process including ESW washing and hydrochloric acid treatment, performed at room temperature, was the same (4.5 h). While the previous report on the transformation of ESW by 5% (w/v) hydrochloric acid treatment included 3×30 min washing followed by 3 h acid treatment [8], the current research included 3×10 min washing and 4 h of 5% (w/v) hydrochloric acid treatment.

Once suspension of ESM completely devoid of the remnants of endogenous calcium carbonate in CaCl_2 solution was obtained, it was subjected to filtration through a 1 mm

pore size plastic screen (Figure 1) in order to separate ESW-derived CaCl_2 solution from ESMs. Separated ESM was subjected to 3×15 min distilled water washing (mass-to-volume ratio: 1:10) in a 3 L glass beaker positioned on an electromagnetic stirrer set at 750 rpm. This was followed by single-volume acetone washing under the same conditions afterward obtained ESM were dried between 18 and 24 h at 60°C in a thermostatic incubator (Heraeus, Germany) and milled using a laboratory mill IKA MF 10.1 (IKA, Staufen, Germany), equipped with a 0.5 mm pore size sieve, at a speed rotation of 4,500 rpm.

ESW-derived CaCl_2 solution obtained after separation from ESM through a 1 mm pore size plastic screen was subjected to two successive steps of vacuum filtration through filter paper Whatman 114 followed by Whatman 1 in order to achieve a clear solution. Afterward, the excess hydrochloric acid was neutralized by the addition of calcium hydroxide, and the solution was left to stand for 30 min for organic matter flocculation. Removal of precipitated organic matter was achieved by vacuum filtration through filter paper Whatman 1. The obtained clear solution of ESW-derived CaCl_2 was then subjected to evaporation to approximately 1/8th of the volume in order to concentrate the solution, and afterward used for the production of CaCl_2 powder by spray drying. It should be pointed out that evaporation of CaCl_2 solution to the desired concentration was not selected arbitrarily but was based on the US 3433863 patent reported by Bowden and Terry [34], where 58% CaCl_2 content in a slurry was found as the most prominent for the production of CaCl_2 powder by spray drying.

Table 2 shows the changes in the CaCl_2 , protein, and amino sugar concentration in the solutions of ESW-derived CaCl_2 during the transformation process, as well as the chemical yield of CaCl_2 and percentage of protein and amino sugar removal. A complete conversion of ESW-calcium carbonate to CaCl_2 by 5% (w/v) hydrochloric acid resulted with CaCl_2 solution of $60.69 \pm 1.05 \text{ g} \cdot \text{L}^{-1}$ containing small amount of proteins ($\gamma = 0.35 \pm 0.04 \text{ g} \cdot \text{L}^{-1}$) and amino sugars ($0.12 \pm 0.01 \text{ g} \cdot \text{L}^{-1}$). Neutralization of the excess of hydrochloric acid with calcium hydroxide followed by organic matter flocculation yielded partially purified CaCl_2 solution of $72.02 \pm 2.93 \text{ g} \cdot \text{L}^{-1}$ from which ~68.6% of proteins and only ~3.7% of amino sugars were removed. Further removal of proteins and amino sugars from CaCl_2 solution occurred during concentration by evaporation to the ~1/8th of volume where in total 99.14% of proteins and 83.25% of amino sugars present in the starting CaCl_2 solution were removed. This can be attributed to the well-known process of the salting out. The data on CaCl_2 and protein concentration in the obtained solution are well in agreement with our previous work [8]. However, there are no data on the

Table 2: Calcium chloride, soluble protein and amino sugar concentration changes during industrial eggshell waste transformation process¹

| Production step | Calcium chloride | | Proteins | | Amino sugars | |
|--|-------------------------------|------------------------|-------------------------------|--------------------------|-------------------------------|--------------------------|
| | γ (g·L ⁻¹) | Yield (%) ² | γ (g·L ⁻¹) | Removed (%) ³ | γ (g·L ⁻¹) | Removed (%) ³ |
| 5% (w/v) HCl solvation | 60.69 ± 1.05 | 100.53 ± 1.56 | 0.35 ± 0.02 | n.a. ⁴ | 0.12 ± 0.01 | n.a. ⁴ |
| Ca(OH) ₂ neutralization and organic matter flocculation | 72.02 ± 2.93 | 99.36 ± 1.24 | 0.11 ± 0.02 | 68.57 ± 4.10 | 0.12 ± 0.00 | 3.71 ± 1.34 |
| Evaporation to the ~ 1/8th of the original concentration of calcium chloride | 590.89 ± 18.92 | n.a. ⁴ | 0.03 ± 0.00 | 99.14 ± 0.46 | 0.19 ± 0.03 | 83.25 ± 1.36 |

¹Results present the mean value ± standard deviations of three independent production batches each performed in triplicate.

²Chemical yield calculated as percentage of ratio of theoretical amount of calcium chloride and experimentally produced amount of calcium chloride.

³Cumulative percentage of removed proteins and sugars from the solution of completely dissolved eggshell waste by 5% (w/v) HCl.

⁴n.a. – not applicable.

concentration of amino sugars in the solution of ESW-derived CaCl₂ so far. Nevertheless, the presence of amino sugars in the solutions of ESW-derived CaCl₂ proved our previous assumption [8] that polysaccharides are probably the major non-proteinaceous organic matter extracted from ESM during the hydrochloric acid treatment of ESW. In fact, this was the main reason why have we monitored amino sugar concentration in the ESW-derived CaCl₂ solutions.

Once a concentrated solution of ESW-derived CaCl₂ was obtained, it was necessary to optimize the process of spray drying in order to produce CaCl₂ anhydride of the lowest moisture content and the greatest yield in the collection beaker. This was done by changing the liquid feed rate from 1.5 to 6.0 mL·min⁻¹, and airflow from 20 to 30 L·min⁻¹. Among five different combinations of liquid feed rate and airflow used for spray drying of a concentrated solution of ESW-derived CaCl₂, the best was the use of liquid feed rate of 1.5 mL·min⁻¹ and airflow of 30 L·min⁻¹ where CaCl₂ anhydride of the lowest moisture content (2.15%) and the greatest yield (91.67%) in the collection beaker was obtained (Table 3). Therefore, the inlet temperature of 210°C,

an airflow on the nozzle of 30 L·min⁻¹, an aspirator rate of 100% (equal to the flow of 35 m³·h⁻¹ of drying air), and a liquid feed rate of 1.5 mL·min⁻¹ were chosen as optimal for spray drying of ESW-derived CaCl₂ of three independent production batches.

The spray drying optimization was also monitored by FTIR-ATR analysis. Figure 4 shows FTIR-ATR spectra of obtained ESW-derived CaCl₂s during spray drying optimization in comparison with commercial CaCl₂ anhydride and CaCl₂ dihydrate. It could be seen that all examined CaCl₂s (ESW-derived and commercial ones) show identical peak positions centered at 1,617, 3,451, and 3,488 cm⁻¹, but differ in their absorbance intensities, where ESW-derived CaCl₂s shows similar peak intensities to the commercial CaCl₂ anhydride. Peaks centered at 3,451 and 3,488 cm⁻¹ can be attributed to the symmetric or asymmetric O–H stretching in crystalline water, and peak centered at 1,617 cm⁻¹ to the H–O–H bending vibration frequency in crystalline water [35].

Table 4 shows the mass yield of ESW-derived CaCl₂ anhydride and ESMs obtained from three independent

Table 3: Optimization of calcium chloride powder production by spray drying of ESW derived concentrated calcium chloride solutions ($\gamma = 590.89 \pm 18.92$ g·L⁻¹) using Mini Spray Dryer B-290

| Drying conditions | Liquid feed rate (mL·min ⁻¹) | Air flow (L·min ⁻¹) | Drying temperature (°C) | | CaCl ₂ in the collection glass beaker | | CaCl ₂ in the drying tower | |
|-------------------|--|---------------------------------|-------------------------|--------|--|------------------------|---------------------------------------|------------------------|
| | | | Inlet | Outlet | Moisture (%) | Yield (%) ¹ | Moisture (%) | Yield (%) ¹ |
| I | 1.5 | 30 | 210 | 146 | 2.15 | 91.67 | 4.59 | 8.33 |
| II | 3.0 | 20 | 210 | 146 | 7.86 | 90.93 | 4.90 | 9.07 |
| III | 3.0 | 30 | 210 | 146 | 6.99 | 90.88 | 4.87 | 9.12 |
| IV | 4.5 | 25 | 210 | 132 | 8.44 | 88.30 | 6.86 | 11.70 |
| V | 6.0 | 25 | 210 | 132 | 8.22 | 80.33 | 11.56 | 19.67 |

¹Yield of calcium chloride in the collection glass beaker and tower is calculated as percentage of ratio of the mass of obtained calcium chloride in the collection glass beaker or drying tower and the sum of masses of calcium chloride obtained in both.

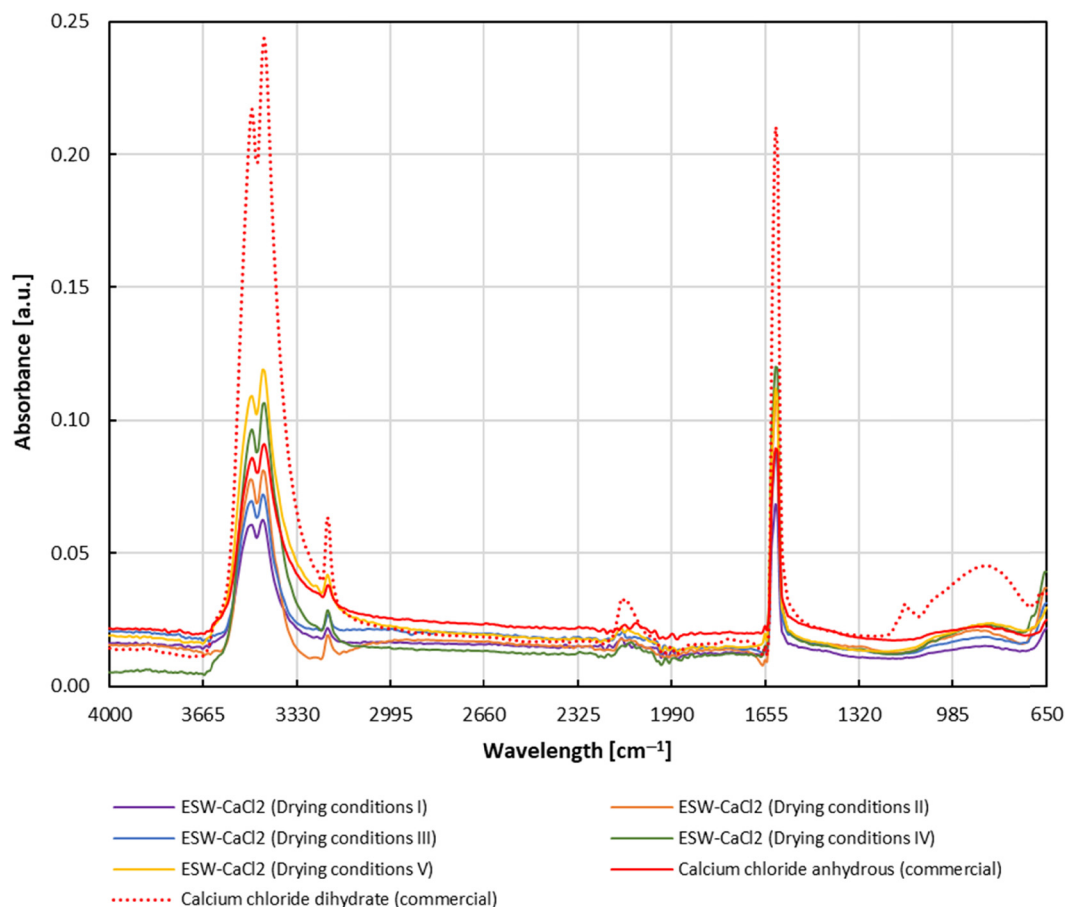


Figure 4: FTIR-ATR analysis of ESW derived calcium chloride in respect to spray-drying conditions. Drying conditions: I – liquid feed rate of $1.5 \text{ mL}\cdot\text{min}^{-1}$; air flow of $30 \text{ L}\cdot\text{min}^{-1}$; II – liquid feed rate of $3.0 \text{ mL}\cdot\text{min}^{-1}$; air flow of $20 \text{ L}\cdot\text{min}^{-1}$; III – liquid feed rate of $3.0 \text{ mL}\cdot\text{min}^{-1}$; air flow of $30 \text{ L}\cdot\text{min}^{-1}$; IV – liquid feed rate of $4.5 \text{ mL}\cdot\text{min}^{-1}$; air flow of $25 \text{ L}\cdot\text{min}^{-1}$; and V – liquid feed rate of $6.0 \text{ mL}\cdot\text{min}^{-1}$; air flow of $25 \text{ L}\cdot\text{min}^{-1}$.

Table 4: Mass yield of calcium chloride anhydride and corresponding eggshell membranes produced from industrial eggshell waste by proposed transformation process

| Transformation product | Mass yield (%) ^{1,2} |
|----------------------------|-------------------------------|
| Calcium chloride anhydride | 102.42 ± 3.31 |
| Eggshell membranes | 2.48 ± 0.28 |

¹Results present the mean value \pm standard deviations of three independent production batches.

²Mass yield calculated on dry weight basis.

production batches. By the use of the proposed ESW transformation procedure, $\sim 102 \text{ g}$ of CaCl_2 anhydrous and $\sim 2.5 \text{ g}$ of ESM can be produced from 100 g of ESW per dry weight basis. According to our knowledge, this is the first report on the possibility of the production of CaCl_2 anhydride from ESW. On the other hand, the possibility of the production of ESM by 5% (w/v) hydrochloric acid treatment of ESW with concomitant production of CaCl_2 dihydrate has

been reported in our previous work [8], with somewhat higher ESM yield of $2.84 \pm 0.16 \text{ g}$ per 100 g of ESW, than those obtained in the current research where $2.48 \pm 0.28 \text{ g}$ of ESM was obtained from 100 g of ESW. The most probable reason for the somewhat lower ESM yield is a difference in the chemical composition of the “industrial” ESW (Table 1) and those collected from households and restaurants [8] attributable to the eggshell color [16,30], laying hen breed, as well as hen housing conditions [32]. Just for example, Kocetkovs et al. [32] reported a significant difference in the ESM thickness between the eggs of different hen breeds and hen housing conditions (cage and cage-free housing).

3.3 ESW-derived CaCl_2 anhydride as a food-grade additive

The ESW-derived CaCl_2 powders were tested on their physicochemical properties, purity, food-grade additive criteria,

Table 5: Physicochemical analysis of eggshell waste derived calcium chloride anhydride

| Parameter | Determined value | Food additive criteria value ¹ |
|---|------------------|---|
| Dry matter content (%) | 98.85 ± 0.01 | n.i.c. ² |
| Calcium content (%) | 35.01 ± 0.63 | n.i.c. ² |
| Calcium chloride content (%) | 96.96 ± 1.74 | ≥93.00 |
| Solubility in water (g·mL ⁻¹) | ≤0.8 | n.i.c. |
| pH of 10% solution | 9.46 ± 0.16 | n.i.c. |
| Free alkali (%) | <0.074 | ≤0.150 |
| Soluble protein content (g·100 g _{d.w.b.} ⁻¹) ³ | 0.05 ± 0.01 | n.i.c. |
| Total protein content (%) | 0.21 ± 0.06 | n.i.c. |
| Amino sugar content (g·100 g _{d.w.b.} ⁻¹) ³ | 0.16 ± 0.01 | n.i.c. |
| Mg and alkali salts content (mg·g ⁻¹) | ≤5.2 | ≤ 50 |
| Mg content (mg·kg ⁻¹) | 1975 ± 361 | n.i.c. |
| F content (mg·kg ⁻¹) | <2.0 | ≤40 |
| As content (mg·kg ⁻¹) | ≤0.031 | ≤3 |
| Pb content (mg·kg ⁻¹) | <0.05 | ≤2 |
| Hg content (mg·kg ⁻¹) | <0.01 | ≤1 |

¹Food Additive Criteria of FAO [38] and EU [37].

²n.i.c. – not included in criteria for food-grade additive.

³d.w.b. – dry weight basis.

as well as microbiological quality criteria. Complexometric titration of obtained powders revealed that ESW-derived CaCl₂ contains 35.01 ± 0.63% of calcium (Table 5), which is slightly lower than the theoretically calculated amount of calcium (36.11%) in CaCl₂ anhydride, indicating that the obtained CaCl₂ is probably in anhydrous form. In this respect, based on the data of complexometric titration, it was found that obtained CaCl₂ powders contain 96.96 ± 1.74% of CaCl₂ anhydride. This was higher than the minimally prescribed content of CaCl₂ (≥93.00) in CaCl₂ anhydride as a food-grade additive (Table 5). Obtained CaCl₂ had somewhat lower solubility in water at room temperature (≤0.8 g·mL⁻¹) than expected (1 g·mL⁻¹) [5], but pH of 10% water solution (9.46 ± 0.16) was quite similar to the pH of 10% water solution of commercial CaCl₂ anhydride (9.65 ± 0.11). Identical solubility and somewhat lower pH of 10% water solution of ESW-derived CaCl₂ dihydrate (pH = 9.34) was reported in our previous research [8]. Since the concentrated solution of CaCl₂ (Table 2) contained a small amount of proteins and amino sugars, it was necessary to examine the content of both in the obtained CaCl₂ powders. ESW-derived CaCl₂ anhydride contained minor amounts of soluble proteins (0.05 ± 0.01 g·100 g_{d.w.b.}⁻¹) and amino sugars (0.16 ± 0.01 g·100 g_{d.w.b.}⁻¹), while total protein content determined by the Kjeldahl method was 0.21 ± 0.06% (Table 5). Somewhat lower amount of soluble proteins (0.02 ± 0.01

Table 6: Crystallographic data of ESW-derived calcium chloride production batches. Data are obtained from Rietveld refinement

| Compound | CaCl ₂ Batch 1 | CaCl ₂ Batch 2 | CaCl ₂ Batch 3 |
|---|---|---|---|
| Space group | | <i>Pnmm</i> | |
| Crystal system | | Orthorhombic | |
| Data collection range (2θ) | | 10–70° | |
| Phase composition (wt%) | | 100 | |
| Z | 2 | 2 | 2 |
| Lattice parameters (Å) | <i>a</i> = 6.2793 (2) <i>b</i> = 6.4229 (2) <i>c</i> = 4.1612 (2) | <i>a</i> = 6.2797 (2) <i>b</i> = 6.4220 (2) <i>c</i> = 4.1598 (2) | <i>a</i> = 6.2786 (2) <i>b</i> = 6.4276 (2) <i>c</i> = 4.1600 (2) |
| Cell volume (Å ³) | 167.8264 (6) | 167.7592 (6) | 167.8807 (6) |
| Calculated density (g·cm ⁻³) | 2.196 | 2.197 | 2.196 |
| No. of parameters refined | 64 | 64 | 64 |
| Average crystallite size (nm) | 26 (2) | 24 (6) | 19 (2) |
| R _B (%) | 7.0 | 6.72 | 6.44 |
| R _p , R _{wp} , R _e (%) | 31.2, 29.8, 19.6 | 28.8, 29.1, 16.6 | 33.5, 30.9, 21.8 |
| GoF | 1.525 | 1.748 | 1.416 |

Legend: numbers in brackets - uncertainty of the last digit (+/-); R_B – Bragg factor; R_p – unweighted profile *R*-factor; R_{wp} – weighted profile *R*-factor; R_e – expected *R* factor; GoF = goodness of fit.

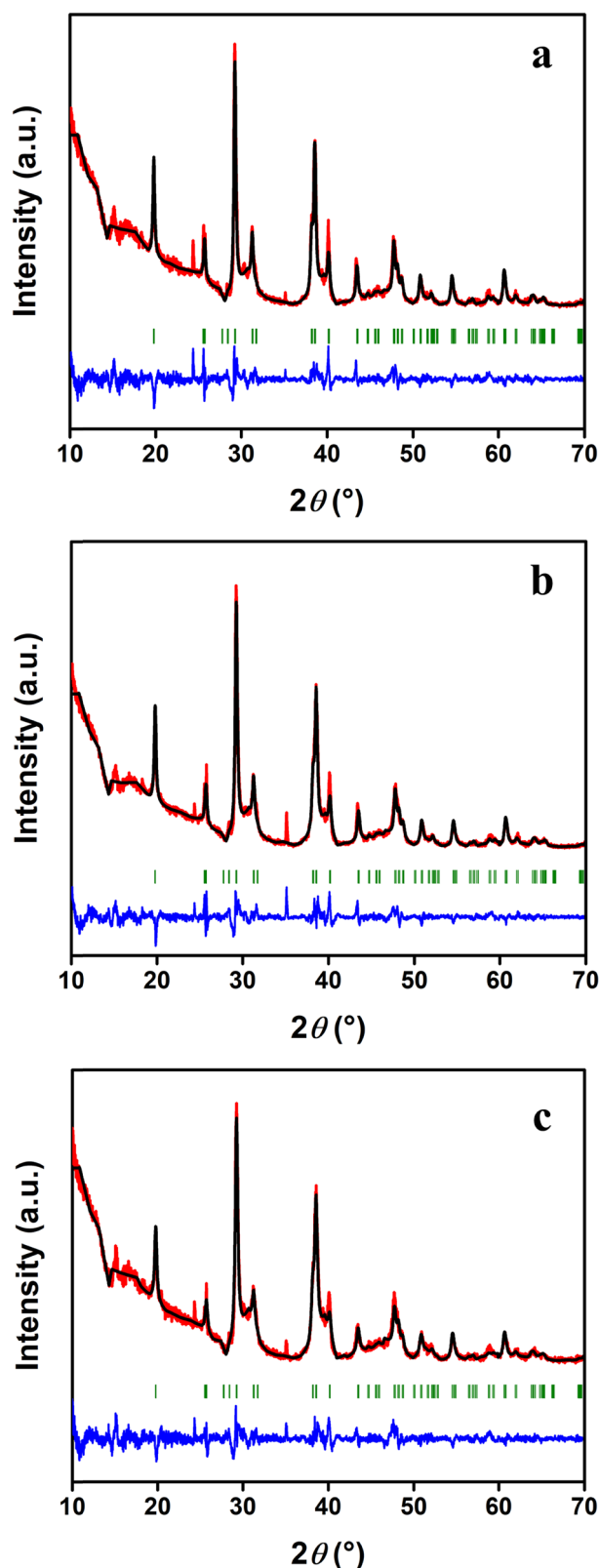


Figure 5: XRD pattern of ESW derived calcium chloride anhydride production batches. (a) The Rietveld plot of the investigated $\text{CaCl}_2 - 1$, (b) the Rietveld plot of the investigated $\text{CaCl}_2 - 2$, (c) the Rietveld plot of the investigated $\text{CaCl}_2 - 3$. The Rietveld plot displays the Bragg reflections and includes the observed (black), calculated (red), and difference (blue) plot, while vertical bars (green) are the expected Bragg reflection positions.

$\text{g}\cdot 100 \text{ g}_{\text{d.w.b.}}^{-1}$) has been reported for ESW-derived CaCl_2 dihydrate powder [8], and a bit higher total protein content (0.29%) in ESW derived $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ [36]. The ESW-derived CaCl_2 fully met all criteria as a food-grade additive prescribed by the EU [37] and FAO [38], as shown in Table 5. Free alkali content was twofold lower than maximally allowed $\leq 0.15\%$, while Mg and alkali salt content almost tenfold (≤ 5.2 vs $\leq 50 \text{ mg}\cdot\text{g}^{-1}$). Moreover, at least 20-fold lower amount than defined maximal values for F, As, Pb, and Hg content in CaCl_2 as a food-grade additive prescribed by the EU [37] and FAO [38] were detected in ESW-derived CaCl_2 anhydride (Table 5). Based on the aforementioned, it can be safely concluded that ESW-derived CaCl_2 anhydride, due to its purity [37,38], obviously has a great potential to be used as a food additive.

In order to confirm the anhydrous form of ESW-derived CaCl_2 powders, their purity, as well as uniformity of the ESW transformation process, XRD analysis of obtained CaCl_2 powders from three production batches was performed. Table 6 shows crystallographic data of ESW-derived CaCl_2 anhydrous analyzed and characterized by the Rietveld refinement of XRD patterns, while Figure 5 shows the Rietveld plots of XRD patterns. Obtained results clearly indicate that all three obtained CaCl_2 powders are identified as anhydrous CaCl_2 that crystallized in the orthorhombic $Pnmm$ space group. Besides Bragg reflections describing anhydrous CaCl_2 (vertical ticks in the Rietveld plot), XRD analysis reveals the presence of some unidentified impurities probably generated during the ESW transformation process. These peaks can be clearly resolved from the peaks of the main phase due to their sharper appearance. It is also worth mentioning that the sharp peak located at 35.1° is attributed to corundum originating from the XRD sample holder. Other crystallographic parameters, such as lattice parameters, cell volume, and calculated density, confirm that obtained CaCl_2 powders are of the anhydrous form [39,40]. In addition, similar values of the crystallographic parameters for each ESW-derived CaCl_2 (Table 6) confirm the uniformity of the ESW-transformation process. In order to visualize the obtained crystal structures, the VESTA software was utilized [41], and atomic arrangements in the obtained CaCl_2 are shown in Figure 6.

Currently, the prescribed criteria for the purity of CaCl_2 as a food-grade additive [37,38] do not involve microbiological quality criteria. This is not surprising since CaCl_2 is mainly produced by hydrochloric acid treatment of limestone. However, the question remains: What if CaCl_2 is produced from an organic source rich in calcium, such as ESW? Is there a need for prescribing microbiological quality criteria for food-grade additives derived from eggs? Considering the fact that egg and egg products are prone to microbiological contamination if improperly

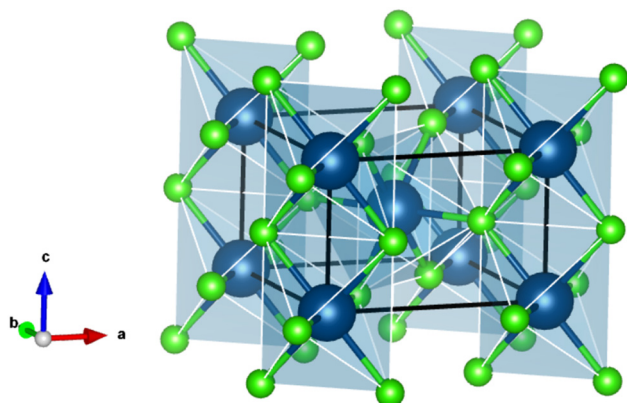


Figure 6: Crystal structure visualization of ESW-derived calcium chloride anhydride. Calcium cation is depicted in dark blue and chloride anion in green.

stored [20,22,42], and the report of EFSA Panel on Dietetic Products, Nutrition and Allergies (EFSA NDA Panel) on the safety of egg membrane hydrolysate as a novel food produced from eggshells [21], it seemed quite reasonable to check the microbial quality of ESW-derived CaCl_2 . In this regard, we have examined the microbiological quality of obtained ESW-derived CaCl_2 powders using the same criteria as those reported by the EFSA panel [21]. The results of examined microbiological parameters in the ESW-derived CaCl_2 powders from three production batches are shown in Table 7. Pathogenic microorganisms including fecal coliforms, *Escherichia coli* and *Salmonella* spp., and *Staphylococcus aureus* have not been detected in ESW-derived CaCl_2 powders. The same situation was in the case of thermophilic spore count and yeasts. Conversely, the presence of bacterial population in all three samples of ESW-derived CaCl_2 powder has been detected by aerobic plate count, while two of the three production batches contained

mesophilic spore (batches 1 and 3) and molds (batches 1 and 2) lower than $100 \text{ CFU}\cdot\text{g}^{-1}$. When obtained data on tested microbiological parameters of ESW-derived CaCl_2 were compared with prescribed microbiological quality criteria (Table 7), it could be seen that two of three production batches, batch 2 and 3, were in compliance with the EU Regulation [21], while production batch 1 fulfilled all criteria except mesophilic spore count ($86 \text{ CFU}\cdot\text{g}^{-1}$) which was higher than prescribed ($\leq 25 \text{ CFU}\cdot\text{g}^{-1}$). Considering the fact that ESW-derived CaCl_2 solution was exposed to high temperature ($100\text{--}150^\circ\text{C}$) during 5 h evaporation, and even higher temperature during spray drying (210°C), it is less likely that elevated mesophilic spore count in CaCl_2 powder from the production batch 1 can be attributed to elevated contamination of starting material (ESW). Rather, it could be a consequence of unintended contamination of powder during handling.

Based on aforementioned, it can be concluded that ESW-derived CaCl_2 due to its acceptable microbiological quality has a great potential to be used as a food additive.

3.4 ESM as a promising carrier for lipase immobilization

Besides a multitude of potential applications in various fields of industry including medical, pharmaceutical, cosmetic, electric, and food industry, well described in reviews of Park *et al.* [43], Shi *et al.* [44], Kulshreshtha *et al.* [45], Mensah *et al.* [46], and Han *et al.* [47], ESM has been reported as a promising platform for the development of immobilized enzyme-based biosensors for glucose [48–51], hydrogen peroxide [52], aspartame [53], homocysteine [54], dopamine [55], urinary oxalate [56], and urea detection [57].

Table 7: Microbiological quality of eggshell waste derived calcium chloride anhydride of three production batches in respect to the prescribed criteria for egg derived products

| Parameter | Prescribed microbiological criteria | CaCl_2 production Batch 1 | CaCl_2 production Batch 2 | CaCl_2 production Batch 3 |
|------------------------------|--|------------------------------------|------------------------------------|------------------------------------|
| Aerobic plate count | $\leq 2,500 \text{ CFU}\cdot\text{g}^{-1}$ | 1,450 | 290 | 1,350 |
| <i>Escherichia coli</i> | $\leq 5 \text{ MPN}\cdot\text{g}^{-1}$ | 0 | 0 | 0 |
| <i>Salmonella</i> | Negative (in 25 g) | 0 | 0 | 0 |
| Coliforms | $\leq 10 \text{ MPN}\cdot\text{g}^{-1}$ | 0 | 0 | 0 |
| <i>Staphylococcus aureus</i> | $\leq 10 \text{ CFU}\cdot\text{g}^{-1}$ | 0 | 0 | 0 |
| Mesophilic spore count | $\leq 25 \text{ CFU}\cdot\text{g}^{-1}$ | 86 | 0 | 5 |
| Thermophilic spore count | $\leq 10 \text{ CFU}\cdot\text{g}^{-1}$ | 0 | 0 | 0 |
| Yeasts | $\leq 10 \text{ CFU}\cdot\text{g}^{-1}$ | 0 | 0 | 0 |
| Molds | $\leq 200 \text{ CFU}\cdot\text{g}^{-1}$ | 15 | 5 | 0 |

CFU – colony forming units; MPN – most probable number.

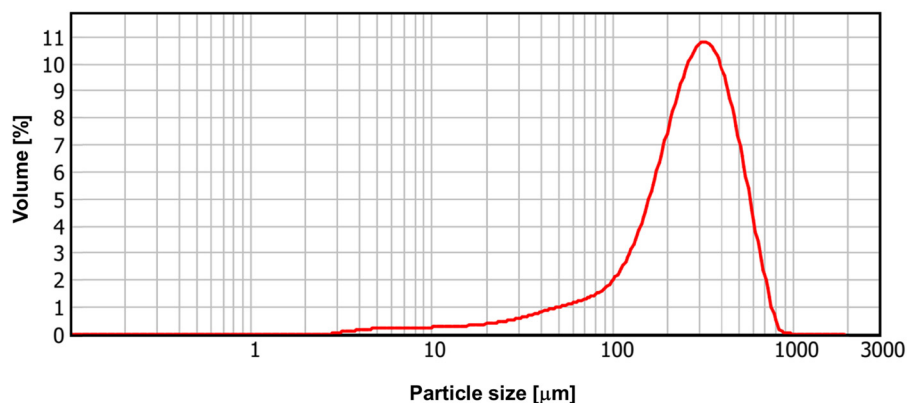


Figure 7: Particle size distribution of ESW derived eggshell membranes. Results are averaged values of three measurements.

However, the majority of aforementioned reports used native ESM for the enzyme immobilization, where ESMs intended for biosensor development were prepared by ESW distilled water washing, stripping off by forceps, and enzyme immobilization performed on ESM pieces ranging in size from 1×1 up to 4×2 cm. This was quite different from the current research, where ESM was obtained by hydrochloric acid treatment of ESW and milled to a particle size less than $500 \mu\text{m}$ (Figure 1) prior to the lipase immobilization. Therefore, it was necessary to investigate whether hydrochloric acid-derived ESM can be used as a promising platform/carriers for enzyme immobilization, especially considering the fact that acid-induced release of ESM from ESW leads to the physical and chemical changes of ESMs [8,9,45–47,58].

The suitability of ESW-derived ESM for enzyme immobilization was examined by the simplest immobilization technique, immobilization by adsorption, using commercial BCL, as the enzyme of interest.

However, prior to the lipase immobilization, it was necessary to confirm that the particle size of the obtained ESM was well in accordance with the desired size range of carriers/beads for enzyme immobilization, as well as to define a minimal volume of enzyme solution, which should be added to the 1 g of dry ESW-derived ESM.

Figure 7 shows the volume-weighted particle size distribution curve of ESW-derived ESM, while Table 8 shows the WHC and OHC of obtained ESMs.

The volume-weighted particle size distribution curve of the ESW-derived ESMs (Figure 7) showed a clear monomodal particle size distribution with a relatively narrow particle size distribution confirmed by a span value of 1.586. The volume-weighted mean diameter of obtained ESMs was $291.89 \mu\text{m}$, with the largest volume fraction of particles in the range of mean particle size diameters from 200 to $400 \mu\text{m}$ (Figure 7) confirming the suitability of

obtained ESM for immobilization. According to the literature, the desirable particle size of enzyme immobilization carrier should range from 20 μm to $500 \mu\text{m}$ [59–62].

Dependent on the extraction method used, it has been reported that ESMs show somewhat different capacities of fluid/water adsorption [58]. In this regard, it was necessary to determine the WHC of ESW-derived ESM obtained by 5% (w/v) hydrochloric acid treatment in order to determine the suitable volume of lipase solution that should be added to ESM during the immobilization process. Besides determination of WHC obtained ESM was also tested on OHC. Corici et al. [60] reported enhanced lipase immobilization on the rice husk when rape seed oil was used for enzyme immobilization in comparison with a buffered enzyme solution.

ESM obtained in the current research showed great WHC of $5.54 \pm 0.12 \text{ mL}$ of water per 1 g of dry ESMs, and almost twice-lower OHC of $3.15 \pm 0.13 \text{ g}$ of olive oil per 1 g of dried ESM (Table 8), indicating high water adsorption capacity of the produced membranes. While the exact data on the oil and water holding capacity of ESM in the currently available literature do not exist, the report of Mensah et al. [58] on ESM water adsorption capacity clearly supports the determined WHC of ESM.

Once WHC of ESM was determined, optimization of BCL immobilization by adsorption onto ESM combining the effect of immobilization duration (from 1 to 6 h) and

Table 8: WHC and OHC of ESW-derived ESMs

| Parameter | Value ¹ |
|---------------------------------------|--------------------|
| WHC ($\text{mL}\cdot\text{g}^{-1}$) | 5.54 ± 0.12 |
| OHC ($\text{mL}\cdot\text{g}^{-1}$) | 3.15 ± 0.13 |

¹Results present the mean value \pm standard deviations of four independent determinations.

lipase activity load (230, 450, 820, 1,190, and 1,430 U) was performed at 25°C. These two parameters of enzyme immobilization have been selected as the most important for defining the suitability of the obtained ESM for lipase immobilization by adsorption due to the significant effect of both on the immobilization efficiency. Immobilization by adsorption of various lipases (commercial or purified ones) onto various hydrophobic and hydrophilic carriers/supports of commercial and/or natural origin, ranging from 30 min up to 20 h, and enzyme activity load between 90 and 50,000 U, with immobilized lipase activities ranging from 0.1 up to 40,000 U per 1 g of the carrier have been reported in the available literature [26,27,60,63–75].

Figure 8 shows the effect of the immobilization duration and commercial BCL activity load on ESM-immobilized BCL activity. One hour of mixing of the suspension of ESM with BCL solutions of different activity loads was found sufficient to obtain maximal non-covalent binding of BCL onto ESM, i.e., immobilization by adsorption, where the maximal activity of lipase of ~124 U per 1 g of wet ESM was obtained by the use of the total activity of lipase of 1,190 U (Figure 8).

The prolonged time of immobilization, greater than 1 h, for all examined activity loads of BCL (230, 450, 820, 1,190, and 1,430 U) resulted in the decreased lipase activity per gram of ESM carriers (Figure 8). This was different from the report of Jiang *et al.* [73], where 8 h of immobilization by adsorption of BCL at the highest activity load of 1,500 U onto ESM has been found to be better than immobilization performed during 0.5 h. However, it should be pointed out that authors used ESM pieces of 1 cm × 1 cm

size instead of milled ones and reported differences in the activity of immobilized lipase by adsorption regarding applied low and high enzyme activity load (300 vs 1,500 U) and low and high time of immobilization (0.5 vs 8 h) were relatively minimal (9.86 ± 0.33 vs 11.52 ± 0.63 mmol·min⁻¹·g⁻¹).

The most probable reason for the observed decrease in the immobilized BCL activity after 1 h of immobilization onto obtained ESM in the current research (Figure 8) is the multilayer adsorption of lipases, which leads to the steric interference of neighboring immobilized enzymes and subsequently hindered activity toward substrate [76,77].

The substantial lack of available literature reports regarding the activity of immobilized lipases onto ESM is one of the greatest obstacles to the actual comparison of currently observed ESM-immobilized BCL activity with previous ones. This is even more pronounced by the fact that various lipase activity assays differ in the determined lipase activity up to 20-fold [28,66,78–81], which additionally disables proper comparison. Nevertheless, ESM-immobilized BCL activity of 124 U per 1 g of wet ESM was found to be greater than those reported by Abdulla *et al.* [74], where 80 U of BCL per 1 g of ESM was immobilized by adsorption followed by glutaraldehyde crosslinking, but lower than reports of Chattopadhyay and Sen [26] and Salleh *et al.* [27] where lipase immobilized by adsorption on eggshells showed activity of ~355 and ~280 U·g⁻¹ of carriers, but with tributyrin as lipase substrate. Regardless, the aforementioned pinpoints ESW-derived ESM by hydrochloric acid treatment as a promising/suitable carrier for lipase immobilization.

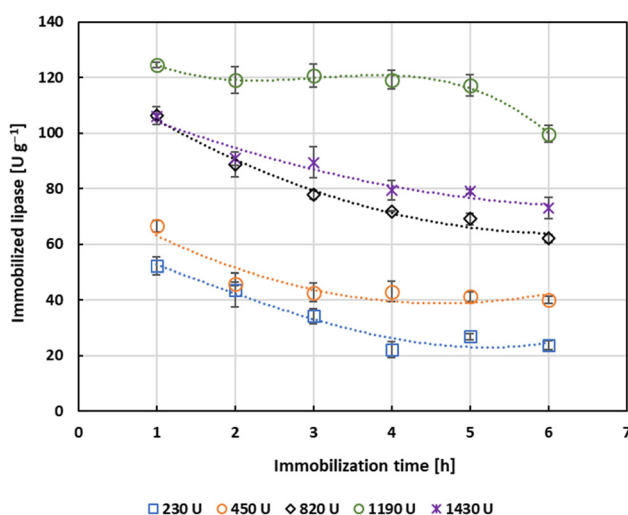


Figure 8: Immobilization of BCL on ESW derived eggshell membranes by adsorption. Results present the mean value ± standard deviations of three independent determinations each performed in triplicate.

4 Discussion

The current research presents an upgraded innovative technique of ESW transformation by 5% (w/v) hydrochloric acid to the value-added products including CaCl₂ anhydride of food-grade additive purity, and ESMs as promising enzyme immobilization carrier (Figure 1), oriented toward *zero waste* model of sustainable waste management strategy. Although it is partially based on the previously reported ESW transformation process by our research group, where ESW collected from households and restaurants was transformed to CaCl₂ dihydrate and ESMs on a laboratory scale [8], here we report upgraded and upscaled ESW-transformation version of the complete process where the majority of previously mentioned future perspectives are fulfilled, and some assumptions proved. This includes the use of “industrial” ESW collected from egg-breaking plants, transformation process scaling-up (7.2-fold), confirmation that part of

glycosaminoglycans have been extracted from ESM during 5% (w/v) hydrochloric acid treatment of ESW at room temperature (Table 2), the replacement of ESW-derived CaCl_2 concentrated solution precipitation with acetone by spray-drying (Table 3), and subsequently omitted necessity of grinding of obtained dried CaCl_2 preparations.

Taken together, it can be safely concluded that the currently proposed ESW transformation process (Figure 1) presents a novel and upgraded version of the possibility of ESW transformation by 5% (w/v) hydrochloric acid to the high-value-added products, as well as ready-to-be-used developed upgraded laboratory transformation process easily transferable to the industrial scale, with obvious possibility of upscaling from the laboratory to the pilot plant scale.

In general, current transformation (Figure 1) of 100 g of ESW on a dry weight basis (or ~118 g ESW on a wet basis) yielded ~102 g of CaCl_2 anhydride of high purity as the main transformation product and ~2.5 g of ESMs as by-product serving as a promising enzyme immobilization carrier (Table 4), which clearly justify the suitability of the proposed transformation process. Aforementioned is even more pronounced if one considers that price of 100 g of CaCl_2 anhydride containing more than 96% of CaCl_2 (ESW-derived CaCl_2 anhydrous contains $96.96 \pm 1.74\%$ of CaCl_2) ranges from 43.20 € up to 182 € [82].

The obtained ESW-derived CaCl_2 anhydride was of high purity and met all prescribed criteria to be used as a food-grade additive (Table 5). Besides prescribed criteria for use as a food-grade additive [37,38], ESW-derived CaCl_2 was tested on its microbial quality criteria (Table 7), simply due to the fact that it was not produced from limestone but from ESW where the necessity for microbial quality testing should be applied [21]. The obtained results (Table 7) have shown the suitability of the proposed ESW transformation process for obtaining microbiologically safe ESW-derived CaCl_2 as a food-grade additive. These findings are even more pronounced if one considers a bit of strengthening EU legislation related to the possibility of reintroducing transformation products derived from by-products of animal origin of category III, such as ESW, back to human consumption [83]. However, it should be pointed out that ESW-derived ESM hydrolysate has been accepted by the EU as a novel food [21], although produced from eggshells. Thus, it seems quite possible that currently obtained ESW-derived CaCl_2 anhydride of high purity has a chance to be considered as a food-grade additive. Nevertheless, it should be noted that CaCl_2 besides its possible use as a food-grade additive has a multitude of alternative uses, including road deicing and stabilization, dust control, concrete curing, oil well drilling, tire ballasting, the production of washing, cleaning and anti-

freeze products, fertilizers and water treatment chemicals [2–5], which clearly justifies the proposed ESW-transformation process. This is even more important if one considers that ~333,525 tons of ESW in the EU are generated by egg-breaking plants annually and the fact that ~118 g of ESW per wet basis ~102 g of CaCl_2 anhydride of high purity can be produced (Table 4). Considering aforementioned, the simplest calculation shows that about 1/3rd of the EU market demands for CaCl_2 of 100,000 tons annually [1] might be ensured by the proposed ESW transformation process using 5% (w/v) hydrochloric acid treatment if it is going to be applied on the industrial scale.

Besides CaCl_2 anhydride as the major product obtained, the proposed ESW transformation process (Figure 1) generates at least two valuable by-products present in much lower amounts (lesser than 3%): adherent egg white protein solution (Figures 1 and 3) and ESMs (Figure 1, Table 4). While the possibility of the use of obtained adherent egg white protein solution was not examined in the current research, ESW-derived ESMs were tested on their suitability to be used as enzyme immobilization carriers. Obtained data (Figure 8) showed a great potential for ESW-derived ESM as BCL immobilization carrier, where up to 124 U of lipase activity per 1 g of wet ESM was achieved with immobilization by adsorption, implying ESM suitability to be used as an enzyme immobilization carrier. The observed is even more pronounced, considering the fact that ESM was found as a promising platform for the production of enzyme-based biosensors [48–58]. Thus, it seems quite possible that the currently proposed production of ESM (Figure 1) might end with the production of ESM pieces suitable for the development of biosensors, omitting the energy-demanding step of ESM milling to a size less than 0.5 mm necessary for successful enzyme immobilization. Nevertheless, the use of ESW-derived ESM for lipase immobilization (Figure 8) justifies the final step in ESM production, a milling procedure, where suitable enzyme immobilization carrier was achieved.

While the suitability of ESW-derived ESM for BCL immobilization by adsorption combining the effect of enzyme activity load and time of immobilization was proved (Figure 8), it should be noted that enzyme immobilization depends not only on the duration of immobilization and enzyme loading but on the other important factors including (a) carrier properties such as particle size, surface area, porosity, pore size, pore volume, and the presence of functional groups necessary for interaction with functional group of enzyme of interest; (b) properties of “free” enzyme such as solubility, pH and temperature stability, enzyme purity, the possibility of aggregation, etc.; (c) the buffer used for immobilization; and (d) pH and ionic strength of the buffer solution [76,84–89]. Therefore,

examination of the applicability of ESM as a promising lipase immobilization carrier using various immobilization techniques is one of the future perspectives.

5 Conclusions

Innovative transformation technique of “industrial” ESW by 5% (w/v) hydrochloric acid treatment to CaCl₂ anhydrous as suitable food-grade additive and ESMs as promising enzyme immobilization carrier, easily transferable to the industrial scale has been proposed. Besides its obvious potential in reducing ESW and subsequent reduction of costs of egg-breaking plants regarding ESW disposal, it clearly offers the possibility of achieving additional profit, if the currently proposed ESW transformation process is upscaled to the industrial scale ending with the production of high-value-added products including food-grade additive CaCl₂ and ESMs as an enzyme immobilization carrier. Although the currently proposed ESW-transformation process might ensure aforementioned, there is still a space for achieving *zero-waste* model approach. This includes the necessity of resolving the fate of adherent egg white protein solutions and its potential application, as well as the possibility of the use of solid residue obtained after organic matter flocculation and subsequent filtration during the preparation of clarified ESW-derived CaCl₂ solutions intended for spray drying. Aforementioned is even more pronounced if one considers that adherent egg white contains valuable proteins such as lysozyme and conalbumin, and that true eggshells (calcified matrix) contain highly valuable antimicrobial proteins which have been obviously precipitated during organic matter flocculation from CaCl₂ solution. Therefore, it seems quite possible that solving currently unresolved issues of the proposed ESW transformation process might lead to the *closing the circle*, i.e., achieving profitable *zero waste* model of ESW transformation.

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