"Recovery of Chitinous Material from Recyclable Waste by Using it in Several Potential Biological Applications (ReWaChi)"

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Abstract

The research undertaken during the implementation of this project led to the following conclusions:

- Recoverable waste for chitin or chitosan from sources such as recycled food residues from shellfish consumption, according to data analyzed from the Food Preferences Questionnaire, can reach approximately 200 g / month / person.
- Preparation both in the private environment and in restaurants can offer the opportunity to collect and capitalize, in short term, a consistent recyclable quantity, in the coastal area, especially in the summer season, per 1000 consumers can be approximated a possible biomass of 54.5 kg / month, of which 10.9 kg is recoverable waste.
- The most important means of assessing and collecting waste from the environment is associated with tracking the effects of release, under the conditions of changing predictable and unpredictable seasonal physicochemical factors with major potential, respectively, the storm.
- Differences in waste frequency are correlated with natural factors (sediment, substrate, species abundance, ecology or biology). The observations shall make it possible to anticipate the achievement of varied biomass and a specific composition for each station and harvest season.
- The most abundant is bio-waste from the natural environment, from the Navodari sector, near the artificial dam, where a wide range of factors is-concentrated, favoring also the level of occasional extraction possibilities or after the storm.
- *R. venosa* provides a considerable mass of organic waste, consisting of empty egg capsules, distinguishing an agglomeration in the upper portions of the supralittoral, respectively the beach accessible to rapid harvesting.
- In the process of obtaining chitin/chitosan from these types of waste, the deproteinization treatment influences the degree of deacetylation, which is maximum when the NaOH concentration is 5% and the processing temperature is low, the conditions of treatment with HCl solution not significantly affecting the degree of deacetylation.
- When both the molar mass of chitosan and the degree of deacetylation are important, then conditions in acid treatment become important. The concentration of HCl may vary within the limits studied, but acid treatment should be carried out in one step when aiming for a chitosan with a high degree of deacetylation and large molar mass, while for a chitosan with a high degree of deacetylation but small molar mass acid treatment should be carried out with more concentrated HCl solution and at least two repetitions. For the latter case, the contribution of the factors "HCl concentration" and "number of acid treatments" is significant.
- Good yields in obtaining chitosan by chemical extraction from R. *venosa* egg capsules are obtained at a temperature of 90 °C, and the concentration of deproteinization solutions, regardless of value, has the same influence. Thus, the extraction process can be considered optimized in terms of yield by using a minimum concentration of NaOH at the processing temperature of 90 °C.
- The evolution of the degree of deacetylation with the variation of the two factors (NaOH concentration and NaOH : chitin ratio (v:w)) shows a continuously increasing variation of DD with increasing both factors. In the investigated field, the maximum value of DD is at the point x1 = 1, x2 = 1 corresponding to a concentration of 55 % NaOH and a ratio v:w of 15:1.

As the experiment carried out at this point led to an average value of 99%, this is practically considered the optimal point.

- It is noted that in terms of NaOH concentration and duration, the conditions for obtaining maximum values for both the degree of deacetylation and the molar mass are very close. The solid liquid ratio, which otherwise has a small influence on these two characteristics of chitosan, seems to be favourable at lower values (15/1) to increase the degree of deacetylation, while the maximum molar mass can be obtained at an 18/1 ratio. Depending on the relative importance of the two criteria (degree of deacetylation and molar mass), appropriate optimal points can be selected from the Pareto front, for which operating conditions can be identified.
- The properties of chitosan nanoparticles, such as size, polydispersity, and zeta potential, depend on both the molecular mass and degree of deacetylation of chitosan and the surfactant used.
- The antioxidant activity of chitosan samples was achieved by monitoring the capture capacity of two types of radicals: ROS (short-lived radicals) by chemiluminescence method and long-lived cationic radicals **ABTS**$^{\bullet+}$ by the TEAC method. Chitosan samples showed ROS radical capture activity ranging from 55.1-98.4% while ABTS radical inhibition capacity$^{\bullet+}$ ranged from 14.33 to 90.9 %, this capacity being influenced by DDA grade and molecular mass of chitosan.
- Chitosan exhibits similar effects in *in vivo* systems at the physiological level, particularly by blocking membrane activity. The effects are influenced by the properties of particles in solutions as well as by the chitin: chitosan: oligochitosan ratio, which influences the passage through membranes.
- Toxicity or cytotoxicity in most tested solutions is reduced or moderate in *Artemia*. Testing on more complex G. *balcanicus* organisms denotes another aspect, namely, the polymer penetration rate at the gill level depends on the chitin: chitosan ratio and generates the modification of ionic homeostasis and the decrease of viability in a short time. The presence of oligochitosan favours the survival of larvae, the penetration of oligochitosan being favored by ingestion.
- Chitosan passes through the digestive tract and is microscopically highlighted in epithelial cells, cuticle, digestive tract cells, as well as in other cell types such as myocytes, after 48 hours after exposing organisms to concentrations of at least 35 μg/mL Cs, the FITC marking method being effective for identifying chitosan in cells.
- Tests to assess the impact induced by these chitosan formulations consisted of analysis of the survival and cell proliferation capacity of normal and tumour cell lines using the colony formation assay (Clonogenic assay) and determination of expression levels of chitinase-like YKL40 protein in order to establish the therapeutic efficacy of these nanocompounds. Epithelial cells from the mammary gland, MCF-12A (ATCC CRL-3598) and tumor cells – epithelial cells from an adenocarcinoma of the uterine cervix, HeLa (ATCC CRM-CCL-2) were analyzed. The SK-MEL-28 melanoma cell line (ATCC HTB-72) was used to evaluate the expression of chitinase-like YKL-40 protein.
- The MTT evaluation of the impact of the chitosan molecules studied in the study on cell viability revealed the induction of a cytotoxic effect, differentiated in amplitude depending on the compound, the dose of *in vitro* treatment – the existence of dose-effect relationship being demonstrated – and the type of cell culture. It is worth noting that in HeLa tumor cell cultures the degree of impaired cell viability is more pronounced than that of healthy MCF-12A cells. Correlated with the results obtained by the MTT test, the impairment of morphology and, implicitly, of cell viability, was more intense in the case of HeLa tumor cells, after the 48-hour treatment.
- All results regarding the evaluation of expression levels of chitinase-like proteins, especially YKL 40 in normal and tumour cell lines, converge to the conclusion that chitosan formulations induced dose-dependent cellular reactivity, demonstrating the existence of dose-effect relationship. Also, the cytophysiological response was influenced by both treatment duration and cell culture type, being more intense after 48 hours of treatment and on the tumor cell line. Expression of YKL-40 (pg/mL) levels in human melanoma, SK-MEL-28 cells after treatment with various chitosan:oligochitosan formulations were manifested by increases reported

in Cs11 and Cs14 variants, slightly higher values compared to the stimulated control. As a result, an increase in expression could be stimulated at low molar mass chitosan concentrations between 400-800 KDa.

- The study included in *in vitro* testing of the impact of chitosan solutions obtained by controlling extraction and purification parameters, so that 14 experimental variants were analyzed and biologically evaluated. Based on the first results obtained in in *vitro* testing (cultures, human cells) and in *in vivo* testing (two biotester organisms, used in toxicity, ecotoxicity, aquaculture applications), new evaluation protocols were created using chitosan mixtures as well as different formulations of chitosan (CH):chitin (CHT):oligomers. Formulations by grafting cerium (Ce) ions onto chitosan were also included in the testing.
- Studies to identify how the biological activity of microorganisms is influenced by the presence of chitosan with different molar mass and in correlation with the variation of concentrations of tested samples, started from the information that the mechanisms which manifested differently on biological systems (bacteria, fungi) are depending on the structure of exposed biological systems and the polymer target at cellular or molecular level (interaction between chitosan and membrane components, how to penetrate into cellular structures). The effects induced by the presence of chitosan molecules with different molar masses and degrees of deacetylation on three strains of bacteria with different structural properties as well as with varied pathogenic and virulent action, strains of interest in the field of public health, were analyzed. Thus, the effects on a gram-positive species *Staphylococcus aureus* (ATCC 23235) were analyzed and from the gram-negative category were evaluated *Pseudomonas aeruginosa (ATCC* 27353) and *Escherichia coli (ATCC 25922*). Two species of the genus Candida were used to test the antifungal action*: Candida albicans ATCC* 10231 and *Candida parapsilosis* ATCC 22019. Chitosan induces inhibition of the bacterium *S. aureus*, depending on the molar mass, thus, in samples with MM between 170 and 413 kDa, effects start at the concentration of 50 μg/mL. Chitosan with MM between 475 and 992 kDa induces effects at concentrations greater than 200 μg/mL. These observations can be linked to explanations in the literature that smaller particles can enter cells faster. No bacterial inhibition phenomena have been identified in chitosan molecules with low molar mass of 26.30 kDa and 155 kDa. The samples tested by chitosan had effects close to bactericidal concentrations of the antibiotics tested, which offers promising prospects for future applications.
- Another aspect analyzed was the adhesion of these microorganisms to chitosan-based films. Thus, membranes obtained from chitosan, of 0.5%, 1%, 2% concentrations, in different solvents (acetic acid and lactic acid), under temperature variation conditions, were exposed to bacterial cultures. It was evaluated how these microorganisms retain adhesion to membrane surfaces or not, which is important for biomedical applications of chitosan, but also in other applications such as obtaining packaging or filters for water. In our study, membranes obtained at low temperatures had a total inhibitory effect at variable concentrations of chitosan on all bacteria. The effect of temperature in the process of setting up membranes is as important as the concentration of the polymer.