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Optimization of chitin yield from shrimp shell waste by *Bacillus subtilis* and impact of gamma irradiation on production of low molecular weight chitosan

Rawia F. Gamal [a], Tarek S. El-Tayeb [a], Enas I. Raffat [b], Haytham M.M. Ibrahim [b], A.S. Bashandy [b,*]

[a] Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Egypt
[b] Department of Radiation Microbiology, National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt

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ABSTRACT

Chitin and chitosan have been produced from the exoskeletons of crustacean shells such as shrimps. In this study, seventy bacterial isolates, isolated from soil, were tested for proteolytic enzymes production. The most efficient one, identified as *Bacillus subtilis*, was employed to extract chitin from shrimp shell waste (SSW). Following one-variable-at-a-time approach, the relevant factors affecting deproteinization (DP) and demineralization (DM) were sucrose concentration (10%, w/v), SSW concentration (5%, w/v), inoculum size (15%, v/v), and fermentation time (6 days). These factors were optimized subsequently using Box–Behnken design and response surface methodology. Maximum DP (97.65%) and DM (82.94%) were predicted at sucrose concentration (5%), SSW concentration (12.5%), inoculum size (10%, containing 35×10^8 CFU/mL), and fermentation time (7 days). The predicted optimum values were verified by additional experiment. The values of DP (96.0%) and DM (82.1%) obtained experimentally correlated to the predicted values which justify the authenticity of optimum points. Overall 1.3-fold increase in DP% and DM% was obtained compared with 75.27% and 63.50%, respectively, before optimization. Gamma-irradiation (35 kGy) reduced deacetylation time of irradiated chitin by 4.5-fold compared with non-irradiated chitin. The molecular weight of chitosan was decreased from 1.9×10^6 (non-irradiated) to 3.7×10^4 g/mol (at 35 kGy).

1. Introduction

Chitin and chitosan are biopolymers of increasing importance and have a significant influence on research in different fields including biology, chemistry, environment protection and health. Their distinguished characteristics interpret the fondness for these natural macromolecules extracted from crustacean shells such as shrimps and crabs, from some fungi, and from insect cell walls [1].

Chitin is a linear polymer of β-1,4-linked *N*-acetyl-D-glucosamine, which looks like the β-1,4-D-anhydroglucopyranose chain of cellulose except for the acetamide group at the C-2 position of an hydroglucopyranoside residue [2]. It is mainly used as a raw material to produce chitin-derived products, such as chitosan, chitooligosaccharides, and glucosamine [3]. Annually, a large quantity of chitin waste is produced from the aquatic food industry; this creates an environmental problem because chitin is a rotting protein [4]. Chitin separation from the crustacean shells is achieved in three steps, deproteinization (DP), demineralization (DM) and removal of lipids and pigments [5]. Currently, DP and DM take place with strong alkali and acids, which have resulted in several serious problems such as hydrolysis of polymer, final inconsistent physiological properties of polymer, and environmental pollution because there is a need for neutralization and detoxification of the discharged wastewater [6]. Alternative and eco-friendly processes to chemical treatment are the utilization of bacterial fermentation and proteolytic enzymes [7]. Based on its natural source and biological function, chitin presented in three different structural forms (α, β, and γ), these forms are differentiated based on the configuration of the carbohydrate chains [8]. β-Chitin exhibits higher

* Corresponding author at: Department of Radiation Microbiology, National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, P.O. Box 29, Nasr City 11731, Cairo, Egypt.
E-mail addresses: monembash@hotmail.com, monembash1@yahoo.com (A.S. Bashandy).

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swelling, solubility, and reactivity compared with α-chitin. Nevertheless, α-chitin is preferentially used in research laboratories and in industries, as it is the major component of the crustaceans' shells [9]. However, chitin still industrially unexploited due to its high degree of crystallinity and insolubility in aqueous and organic solvents [10].

On the other hand, chitosan, the deacetylated form of chitin, is a high molecular weight cationic polysaccharide consisting of (1-4)-2-amino-2-deoxy-β-D-glucan [11]. The molecular weight and the degree of deacetylation are the most important physicochemical properties of chitosan since these parameters determine the quality of chitosan in its diverse applications [12]. The degree of acetylation is described by the percentage of acetylation or the molar fraction of *N*-acetylated units. When the degree of acetylation is lower than 50%, chitosan becomes soluble in acidic aqueous solutions due to protonation of NH_2 group at the C-2 position of glucosamine units [13]. Based on molecular weight, chitosans can be classified into high molecular weight-, low molecular weight-, and oligochitosan [14]. There is a correlation between the bactericidal activity of chitosan and its molecular weight. High molecular weight chitosan molecules can not penetrate the microbial cell membrane, they accumulate on the cell surface and block nutrient transport leading to cell lysis [15]. In contrast, low molecular weight chitosan molecules (<5000 kDa) dissociated in solution, could penetration the nuclei of the microorganisms, bind with DNA and inhibit synthesis of mRNA [16]. Chitosan has been demonstrated to be biodegradable, non-toxic, biocompatible, and has a broad-spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria, yeasts as well as fungi [17,18–20]. It has many applications in pharmaceutical, medicine, biomedical, agriculture, biological, environment, and in food industry which involves antimicrobial packaging film, food formulations, binding, gelling, thickening, clarifying, stabilizing, and antimicrobial agent [21–23]. Dehand et al. [13] developed a chitosan–nanocellulose nanocomposite from high molecular weight chitosan and nanocellulose particles. The optimized nanocomposite films revealed outstanding mechanical properties as compared to some synthetic films, and had inhibitory effects against some food pathogens namely, *Staphylococcus aureus*, *Escherichia coli,* and *Salmonella enteritidis* bacteria. Similarly, Benhabiles et al. [24] established the environmental and economical advantages of using chitosan coatings to improve the quality and storability of strawberries, and extend the shelf life, by reducing weight loss, delaying senescence, retaining brightness, soluble solids content, and titrable acidity content.

Response surface methodology (RSM) is useful software for designing experiments, building models and analysing the influences of numerous independent variables. RSM can reduce the number of experimental trials, determine the significant reaction factors, and can be employed to optimize the treatment conditions and processes [6].

The aim of this work was to produce low molecular weight chitosan as a part of the extraction process from shrimp shell waste (SSW). Therefore, the influence of several operating parameters including the carbon source, SSW concentration, inoculum size and fermentation time on the DP and DM degree of SSW, by a locally isolated *Bacillus subtilis* strain, was investigated. Response surface methodology (Box–Behnken design) was employed to study the interactions between the selected reaction variables and the degree of DP and DM. Analysis has been used to optimize the experimental conditions allowing maximum DP and DM degree. The effect of gamma radiation on the reduction of deacetylation time of chitin and the molecular weight of obtained chitosan was also investigated.

2. Materials and methods

2.1. Sources of bacteria

All the protease producing bacterial isolates used in this study were isolated from the rhizosphere of different cultivated plants. The proteolytic *Bacillus subtilis* -262 [25], obtained from NCRRT, was used as a standard strain. The bacterial strains were maintained on nutrient agar slants at 4 °C and subcultured at monthly intervals.

2.2. Shrimp shells waste (SSW) preparation

SSW was obtained from Al-Oboor fish market, Cairo, Egypt. All shells were equal in size and from a single species of shrimp (*Parapenaeus longirostris*). The shells were kept frozen during transportation and preservation until processing. The shells were soaked in a chlorine solution (1%, v/v) for 30 min, to remove any impurities, and washed with tap water several times to get rid of the residual chlorine, then, dried in an oven at 50 °C overnight to breakdown the crystalline structure of chitin and to make them more tenuous. Dried SSW was preserved at 4 °C in tightly closed plastic bags.

2.3. Bioprospecting of proteases producing bacteria

Isolation of different bacterial species capable of growing on SSW, as the sole carbon and nitrogen source, was achieved by employing the pour plate method. Shrimp shell agar medium [26], containing (g/L): shrimp shell powder 30, K_2HPO_4 1, $MgSO_4{\cdot}7H_2O$ 0.5, agar 20, at pH 7.0 ± 0.2, was used. Ten grams of each soil sample and shrimp shell waste were transferred to 250-mL conical flasks containing 90 mL of sterile saline solution. Flasks were shaken well for 15 min. Then, the mixtures were serially diluted up to 10^8 in sterile saline solution. Aliquots of appropriate dilutions (0.1 mL) were inoculated into 10 mL of melted shrimp shell agar in triplicates and mixed well before solidification. Plates were incubated at 35 °C for 48–72 h. The developed colonies were picked up, purified, and maintained on nutrient agar slants at 4 °C. Later, the isolates were tested for proteases enzymes production utilizing skim milk agar medium [27]. Protease production was expressed as the diameter of developed clear zone (cm).

2.4. 16S rRNA sequencing analysis

The most efficient proteases producing isolate was identified according to the morphological characteristics (shape of cells, Gram staining, motility, and shape of colonies), and confirmed by the 16S rRNA gene sequence analysis. The bacterial whole genome was extracted using Wizard genomic DNA isolation kit (#A1120, Promega Corporation, USA). Amplification of the 16S rRNA gene was carried out using PCR (green master mix, Promega Corporation, USA), and employing 8F as a forward primer 5′ AGA GTT TGA TCC TGG CTC AG 3′ and U1492R as a reverse primer 5′ GGT TAC CTT GTT ACG ACT T 3′. The sequence similarity was compared with the Gene Bank data bases using BLASTIN software by the Finch TV programe (http://www.geospiza.com/Products/finchtv.shtml).

2.5. Factors affecting chitin production from SSW by B. subtilis

The fermentation medium described by Sini et al. [28], containing (g/L): brown sugar 20, shrimp shell 20, at pH 7.0 ± 0.2, was subjected to several optimizations in order to attain the highest deproteinization (DP) and demineralization (DM) degree. To study the effect of carbon source, brown sugar was replaced with sucrose, glucose, and fructose, at the same concentration. In 250-mL Erlenmeyer flasks, 100 mL portions of the fermentation medium, containing each of the tested carbon sources individually, were