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Synergistic effect of chitosan immobilized L-asparaginase nanobiocomposite on acrylamide mitigation in fried bitter gourd chips

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One of the major challenges in food industries is to remove acrylamide present in the fried foods. Asparaginase is generally used to mitigate acrylamide in fried food. Here, we studied the synergistic effect of chitosan with asparaginase on mitigation of acrylamide in fried bitter gourd chips. The chitosan immobilized asparaginase (CIA) nanobiocomposite was characterized using AFM. The treated and untreated fried chips were characterized using SEM and FT-IR analysis. The optimized pre-treatment temperature of 55ºC and 10 min time showed less acrylamide formation using CIA nanobiocomposite. The acrylamide formation was reduced to 948 µg/kg at 170ºC frying temperature for 5 min using 3 U/mL of CIA nanobiocomposite. The use of CIA nanobiocomposite was found effective for mitigation of the acrylamide present in fried bitter gourd chips. Chitosan provided synergistic effect with asparaginase on acrylamide mitigation and it neither altered the appearance nor the taste of the fried chips.

Keywords: Acrylamide mitigation, Fried foods, Food Processing, Immobilized asparaginase, Nanobiocomposite, Nesslerization QuEChERS extraction

Fried and baked foods are the main reservoirs for the onset of several diseases such as obesity, high cholesterol leading to coronary heart disease, including life threatening diseases like cancer. The indigenous contaminants and components formed during heating of starchy foods at high temperature during the preparation of fried and baked foods induce human cells to proliferate rapidly and lead to cancer. Acrylamide is a compound present in starchy food products such as potato chips, french fries, biscuits, bread and wide range of other heat-processed food products[1,2]. Acrylamide has not been identified in unheated or boiled foods. Acrylamide has been declared as a highly carcinogenic compound in humans, so it is important to reduce its level in foods as recommended by the FDA. The amino acid (asparagine) and monosaccharides (fructose and glucose) present in starchy foods are converted into acrylamide during heating at high temperatures[3-6]. The mechanism of Maillard Reaction is not depicted clearly as the perception towards food products changes with respect to processing technology[7].

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The toxic effect of acrylamide was investigated later when humans were exposed to this organic compound occupationally. Number of studies were reported the dietary effect of acrylamide in the major risk of development of cancer in mostly humans. The relative risk factor of cancer was calculated with the rise of 10 µg/day to the other people those who are detected with cancer[2,8]. Research studies reported that the higher risks of acrylamide intake were related with ovaries, endometrial cancers, renal cancer and oesophageal cancer. Various bioassay of the acrylamide injection in mice were evaluated, which showed positive results on increase in chance of sub-type of ovarian cancer[9].

Different formulation and processing strategies are used to reduce acrylamide in various thermally processed food products. Different strategies are used for acrylamide mitigation in food products that were based on raw material effects, additives, processing conditions (Time, Temperature, pH, water and fermentation). The further research and cooperation among food scientist, manufacturers and regulatory organization is important in mitigation of acrylamide in thermally processed food products. The FAO/WHO Expert Committee on Food Additives has suggested asparaginase to reduce acrylamide formation during

processing of high-starch containing food products[10,11]. Hence, it is important to develop novel and effective pathways to lower the severity of acrylamide formation during heating of starchy foods at high temperature. Acrylamide formation during frying can be reduced by washing starchy foods before frying using asparaginase solution as it cleaves asparagine. Asparaginase acts as a catalyst and converts the asparagine into ammonia and aspartic acid[3,4,12].

The high-pressure technology with asparaginase for acrylamide mitigation in raw potato sticks reported 26-47% of acrylamide reduction. This process is nonthermal and low-cost technology for acrylamide mitigation[13]. The novel type-I asparaginase from the microorganism *Acinetobacter soli* was used to reduce the acrylamide level in fried potato chips. The sample with enzyme treatment showed 55.9% acrylamide reduction[14]. L-asparaginase from *Aspergillus terreus* BV-C strain, utilizing flaxseed oilcake achieved a notable 93% reduction rate in acrylamide levels demonstrating its efficacy for acrylamide mitigation[15]. Asparaginase immobilized on magnetic nanoparticles was reported better acrylamide mitigation[16].

In this study, we have made an attempt to use fungal asparaginase as pretreating agent for preparation of bitter gourd fried chips. Different parameters such as sample size, asparaginase concentration (both free and chitosan immobilized), soaking time, frying time and specific temperature on acrylamide mitigation was studied and optimized. The changes in physicochemical properties of fried bitter gourd chips were characterized using FT-IR and SEM analysis.

Materials and Methods

L-asparaginase production using *Aspergillus terreus*

The L-asparaginase was produced from *Aspergillus terreus* MTCC 1782 obtained from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. To facilitate L-asparaginase production, a modified Czapek-Dox liquid medium was employed. Following fermentation, the fungal biomass was separated by filtration using Whatman #2 filter paper. The resulting clear, homogeneous filtrate containing the crude asparaginase enzyme was collected for further use[17].

Assay of asparaginase activity

The Nesslerization method was to determine the activity of crude asparaginase and chitosan immobilized asparaginase[18].

Synthesis of asparaginase immobilized chitosan nanobiocomposite

The chitosan powder (0.01% w/v) was dissolved in a solution containing 0.05% acetic acid. The mixture was stirred continuously for 1 h to ensure complete dissolution and homogeneity. After dissolution, 1 M NaOH was added to the chitosan solution, resulting in the formation of a precipitate. The precipitate was separated by centrifugation at 10,000 rpm for 10 min and washed thoroughly with distilled water to remove any impurities. The washed chitosan precipitate was treated with 1% glutaraldehyde at 32°C for 3 h. This step facilitated the cross-linking of chitosan molecules, providing a stable matrix for enzyme immobilization. Following glutaraldehyde treatment, the precipitate was washed with Tris-HCl buffer (50 mM, pH 8.5) to remove any unreacted glutaraldehyde, ensuring the integrity of the immobilization matrix. The prepared chitosan particles were mixed with L-asparaginase and incubated at 32°C for 30 min to allow for enzyme immobilization onto the chitosan matrix. After immobilization, the mixture was subjected to centrifugation at 10,000 rpm for 10 min to separate the chitosan immobilized L-asparaginase (CIA) nanobiocomposite from the solution. The resultant CIA nanobiocomposite was utilized for acrylamide mitigation in fried bitter gourd chips[19-24].

Pre-treatment of bitter gourd slices

The bitter gourd samples were sliced into uniform sizes and washed using distilled water. Slices were pretreated using free asparaginase and CIA nanobiocomposite. Further the treated slices were washed using distilled water for completely removing the presence of enzyme and CIA nanocomposite residues.

Optimization of pre-treatment and frying conditions

The sliced and untreated bitter gourd were soaked in fungal asparaginase solution and additives. The soaking time, size of untreated bitter gourd, concentration of enzyme, soaking temperature was varied and its effect on acrylamide mitigation was studied and optimized in order to achieve higher acrylamide mitigation. The asparaginase treated bitter gourd was water washed and dried to remove aspartic acid and ammonia formed due to the action of asparaginase on asparagine and sugars present in untreated foods. Asparaginase pre-treated and washed bitter gourd was heat processed at various conditions such as at different time of frying, oil used for frying, without oil (air frying) and temperature of frying on acrylamide mitigation and optimized. Acrylamide

content in fried bitter gourd without pre-treatment and with pre-treatment using asparaginase and frying was estimated using HPLC. The acrylamide mitigation effect of CIA nanobiocomposite was studied under optimal conditions by treating the starchy food products using immobilized asparaginase.

Extraction and estimation of acrylamide from bitter gourd chips

Agilent QuEChERS method is found to be the best for extraction of acrylamide from bitter gourd. In this method, powdered fried chips weighted 1 g are spiked with the internal standards and mixed vigorously for 1 min. The two solvents acetonitrile and n-Hexane with 2:1 ratio was used for acrylamide extraction along with the use of 9 ml HPLC grade water, followed by additives or divalent cations such as 4 g of $MgSO_4$ and 0.15 g of NaCl were mixed together and then centrifuged at 4000 rpm for period of 5 min. Then after centrifugation, hexane solvent present in upper layer was removed. The 6 ml bottom solution was properly mixed along with addition of PSA, C18EC and $MgSO_4$ 755 *g* followed by centrifugation for 5 min. The extracted solution (1000 µL) used for acrylamide estimation using HPLC (Agilent 1260 Model, Agilent Technologies, Germany)[25]. The ZORBAX HILIC plus Column of (4.6 mm × 50 mm × 3.5 µm) with Agilent Chemstation for LC/MS 2D software was used.

Characterization of CIA nanobiocomposite and fried bitter gourd chips

The size and surface morphology of the synthesized CIA nanobiocomposite was analysed using AFM. The morphological changes and surface texture characteristics of fried bitter gourd chips before pretreatment and after pretreatment was analysed by SEM and FTIR[26].

Results and Discussion

Mitigation of acrylamide in bitter gourd chips

Effect of pretreatment temperature and time

The study on the effect of various parameters including concentration of the free chitosan, free enzyme and also chitosan immobilized with asparaginase, and pretreatment conditions were studied. The soaking temperature varied from 40 to 60ºC and time varied in the range of 10 to 20 min with intervals of 5 min. The optimized pretreatment temperature of 55ºC and time 10 min showed low acrylamide formation (1686 µg/kg) in bitter gourd chips (Fig. 1). The untreated sliced bitter gourd had an average size of 35 mm and a thickness of 4 mm, with no change observed post-treatment.

Effect of free enzyme and chitosan immobilized with asparaginase nanobiocomposite

The study on the effect of free enzyme and chitosan immobilized with asparaginase nanobiocomposite on acrylamide mitigation in bitter gourd chips is shown in Fig. 2. The free enzyme and the chitosan immobilized with asparaginase were varied with the concentration from 1 to 6 U/mL at optimized frying temperature of 170ºC. The minimum acrylamide concentration 1872 and 1122 µg/kg were obtained at 5 U/mL of free and chitosan immobilized asparaginase, respectively.

Effect of the frying temperature and frying time

The acrylamide mitigation was analysed based on various frying temperature and time. Time and temperature play a crucial role in reducing the

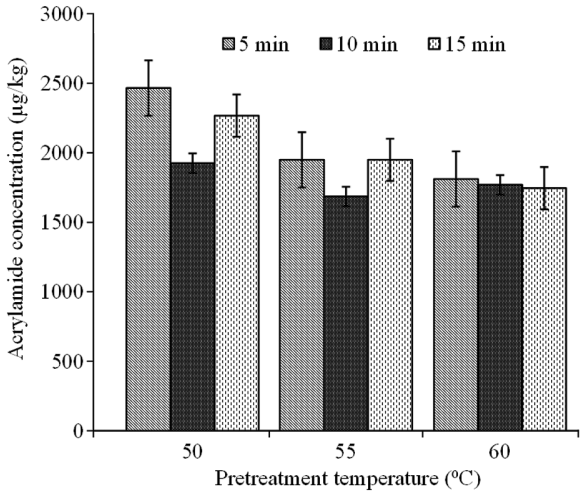

Fig. 1 — Effect of pre-treatment temperature and time on acrylamide mitigation in fried bitter gourd chips

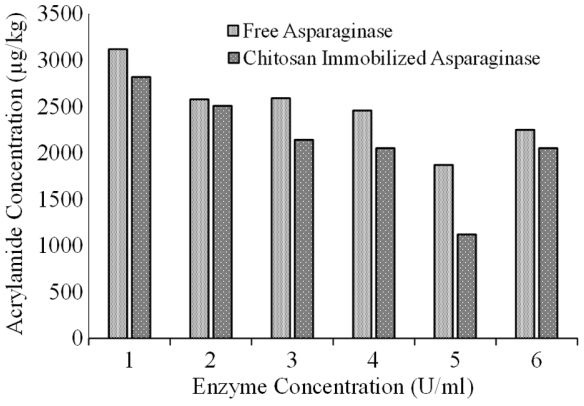

Fig. 2 — Effect of free and chitosan immobilized asparaginase nanobiocomposite

concentration of the acrylamide hence, the frying temperature and the time was studied for free enzyme asparaginase (Fig. 3A), free chitosan (Fig. 3B) and chitosan immobilized with asparaginase nanobiocomposite (Fig. 3C). The soaked bitter gourd (55ºC at 10 min) was used in this optimization study. The frying temperature (160-180ºC) and time (5-10 min) varied and optimized. In free asparaginase, minimum concentration of acrylamide (1524 µg/kg) was obtained at 170ºC which is fried for 10 min. In free chitosan, minimum concentration of acrylamide (2010 µg/kg) was obtained at 170ºC which is fried for 10 min. In chitosan immobilized with asparaginase, minimum concentration of acrylamide (960 µg/kg) was obtained at 170ºC for 10 min. In the overall process of optimization, chitosan immobilized asparaginase has showed significant effect on mitigation of acrylamide.

Characterization of chitosan immobilized asparaginase nanobiocomposite using AFM

The AFM image analysis was used to study the size and morphological structure of synthesized CIA nanobiocomposite. The analysis was performed on scanning area 2×2, 3×3, 5×5 and 10×10 µm^2. Same scan rate (1 Hz) was used for all measurement with same ambient conditions (set point-14.8 nm, amplitude-20.21 nm, Frequency-311.47E3 Hz, Drive-29%). The average size of the CIA nanobiocomposite was found as 26.18 nm. The roughness surface of the chitosan immobilized asparaginase shows in Fig 4. The rough morphological structure and high surface area shows the significant impact on lowering acrylamide content in chips.

Characterization of fried bitter gourd chips

The surface morphological characteristics of bitter gourd chips were analysed using obtained SEM images. The SEM images of untreated and treated fried bitter gourd chips were shown in Fig. 5(A & B). The untreated chips were shown rough and non-porous surface. The treated chips were shown smooth and porous surface. The chitosan immobilized asparaginase treated chips was shows homogeneous morphological surface. The FT-IR analysis of bitter gourd chips was shown in Fig. 6. The FT-IR analysis provides the occurrence of functional groups present in the bitter gourd chips. The intensity of the amine groups (N-H) in treated chips was lower than the untreated chips. The chitosan immobilized with

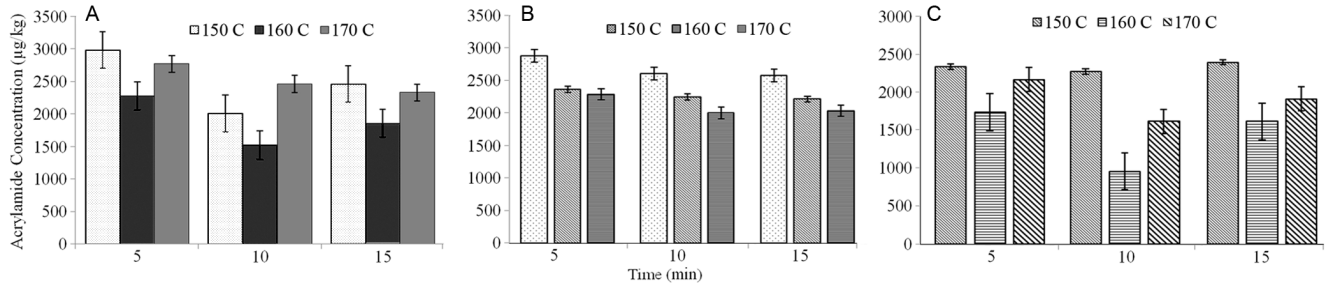

Fig. 3 — Effect of frying temperature and time using (A) free asparaginase; (B) free chitosan; and (C) chitosan immobilized asparaginase nanobiocomposite

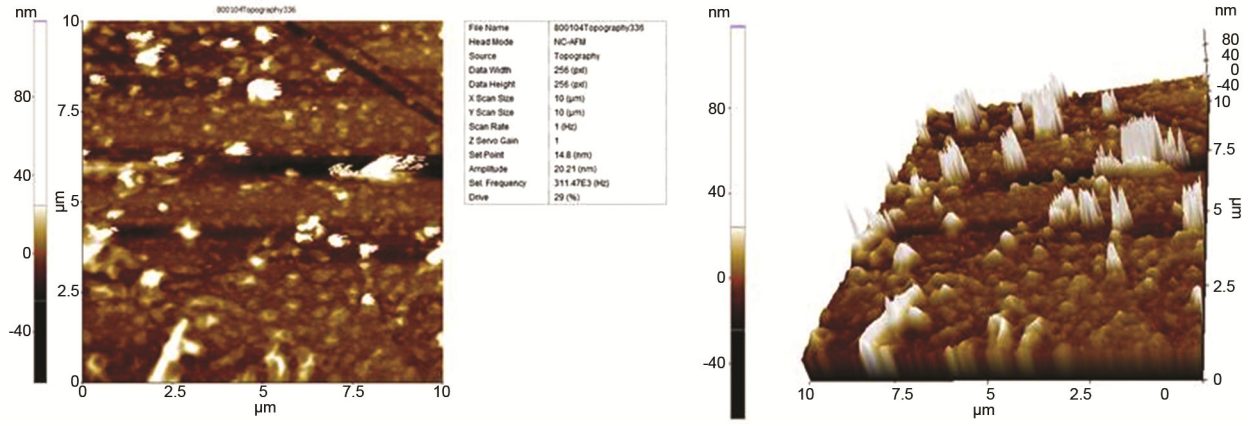

Fig. 4 —. AFM image of chitosan immobilized asparaginase

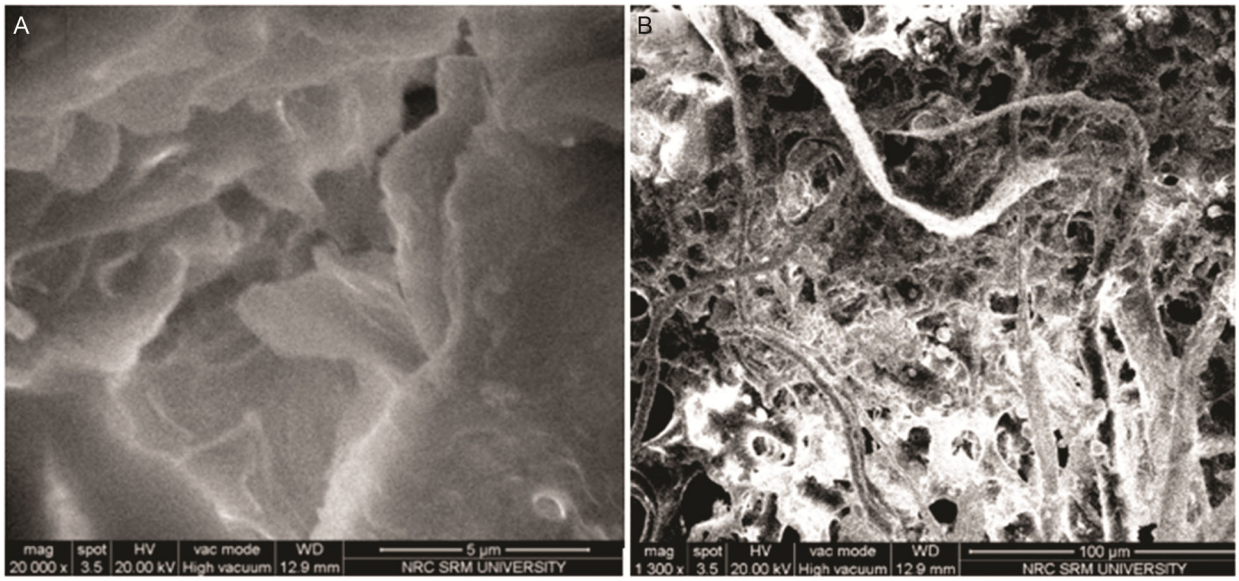

Fig. 5 — SEM image of fried bitter guard chips (A) without treatment; and (B) with chitosan immobilized asparaginase treatment

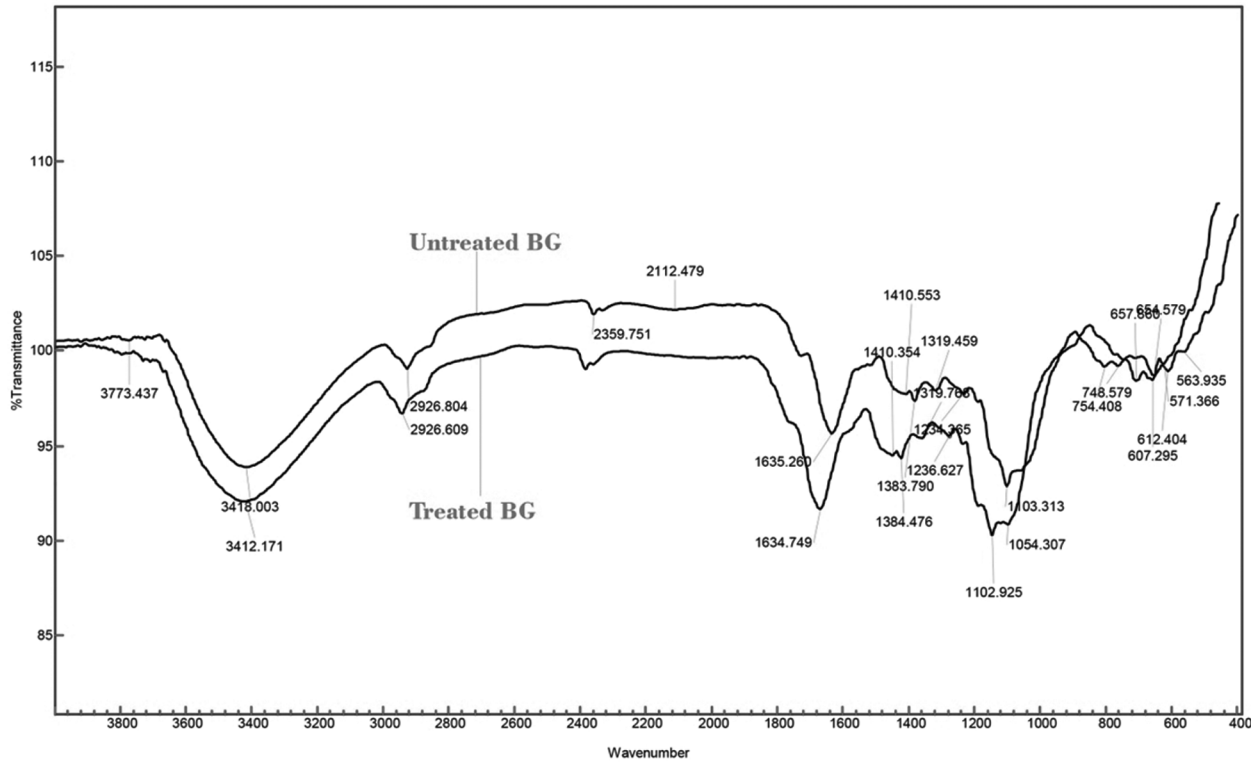

Fig. 6 — FT-IR analysis of untreated and treated bitter gourd chips

asparaginase shows better mitigation of acrylamide in frying process. The immobilized chitosan with asparaginase was found more highly effective in mitigating the concentration of the acrylamide in bitter guard chips due to the synergistic effect of the chitosan with asparaginase. Hence the cost of the acrylamide mitigation process will become cheaper and socially acceptable even for house hold applications and industries.

Conclusion

The acrylamide level in bitter gourd fried chips was reduced using asparaginase highlighting that neither altered the appearance nor the taste of the final

product. The chitosan which was immobilized with asparaginase has significant effect in mitigating the concentration of the acrylamide in bitter gourd chips due to the synergistic effect chitosan with asparaginase. Hence, the cost of acrylamide mitigation process will become cheaper and socially acceptable even for house hold applications by housewife and road side chips shops.

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Conflict of interest

Authors declare no potential competing interests.

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