



Estimation of Free and Immobilized Protease Efficiency in Inhibiting of Food Spoilage Bacteria

Mohammed Abdulrazzaq Alsoufi¹, Raghad Akram Aziz²

10.18805/ag.DF-615

ABSTRACT

Background: An immobilized enzyme has been meeting the demands of clean usage technologies and sustainable production practices in food sciences applications.

Methods: Partial purification of protease from Prickly lettuce *Lactuca serriola* L. leaves using ammonium sulfate, immobilization, characterization and application as antimicrobial against the growth of some food spoilage bacteria, either alone or in synergistic with extract of Welsh onion *Allium fistulosum* L.

Result: The immobilization efficiency of protease was 78%. The optimum pH and temperature of activity for free (FP) and immobilization (IP) protease was 8.5 and 60°C, respectively. The (FP) and (IP) were stable for 4 and 15 days of storage at 4°C. The (IP) activity was stable up to 15 reuses. The antimicrobial effect of (IP) was greater than that of (FP) on *Staphylococcus aureus* and *Escherichia coli*, while it was the opposite for the use of Welsh onion extract (WO). The synergistic effect from (IP) and (WO) (1:1) showed a noticeable increase in the inhibition growth of both bacteria. The absorbance (%) at 610 nm for growth of bacteria over incubation showed significant change ($P \leq 0.01$) compared to the control to be 22.48 ± 1.37 , 45.74 ± 2.06 , 33.33 ± 1.79 , 63.75 ± 3.64 and $82.17 \pm 4.05\%$ for *S. aureus*, while it is 9.52 ± 0.71 , 68.71 ± 3.52 , 45.59 ± 2.75 , 77.55 ± 3.81 and $92.52 \pm 3.98\%$ for *E. coli* when using 100, 100, 75+25, 25+75, 50+50 mg of (IP) and (WO), respectively.

Key words: Antimicrobial activity, Enzymes applications, Food spoilage, Immobilized enzymes, Protease, Welsh onion extract.

INTRODUCTION

Enzymes in industrial production and other applications will meet the requirements for clean usage technologies and sustainable production steps, resulting in low environmental effects, costs, energy, water, chemicals and time use in production steps. Thus, research has increased to find enzymes that have high stability at pH, temperatures and storage and are reusable (de Oliveira *et al.*, 2021). Protease is a type of enzyme that belongs to the hydrolase group and has wide physiological roles in the growth of animal, plant and microorganism cells and differentiation (Abdullah *et al.*, 2022; Qamar *et al.*, 2020). It also has critical applications in the food and feed industry, improving the properties of proteins, analyzing proteins, pharmaceutical and medical preparations, cosmetics, textile and leather, detergents, effluent treatment and protein engineering (Fguiri *et al.*, 2023; Mirza *et al.*, 2023; Dinani *et al.*, 2021; Al-Soufi, 2013).

The effect of proteolytic enzymes as antimicrobial alone or synergy with plant-derived compounds or antibiotics was reported by several authors, such as effective against *Listeria monocytogenes*, *Bacillus* sp, *Pseudomonas* sp, *Staphylococcus simulans* and *Escherichia coli* ATCC 25922 (Salinas Ibáñez *et al.*, 2021), *Bacillus subtilis* and *Staphylococcus aureus* (Indarmawan *et al.*, 2016); *Acinetobacter* sp. KC119137.1 and *S. aureus* NCIM 5021 (Manohar *et al.*, 2015). Currently, immobilized enzymes are considered a promising technology in enzymology that aims to be less wasteful in using free enzymes due to the relatively high costs of production (Guler *et al.*, 2020). The benefit of immobilization is represented by the reuse of

¹Department of Products Evaluation and Service Performance, Market Research and Consumer Protection Center, University of Baghdad, Baghdad, Iraq.

²Department of Science, College of Basic Education, Mustansiriyah University, Baghdad, Iraq.

Corresponding Author: Mohammed Abdulrazzaq Alsoufi, Department of Products Evaluation and Service Performance, Market Research and Consumer Protection Center, University of Baghdad, Baghdad, Iraq.

Email: alsoufim@mracpc.uobaghdad.edu.iq

How to cite this article: Alsoufi, M.A. and Aziz, R.A. (2024). Estimation of Free and Immobilized Protease Efficiency in Inhibiting of Food Spoilage Bacteria. Agricultural Science Digest. 1-9. doi: 10.18805/ag.DF-615.

Submitted: 12-04-2024 **Accepted:** 10-10-2024 **Online:** 14-12-2024

enzymes many times without any loss in activity and the ability to complete the required reaction (Alsoufi, 2021). Therefore, protease immobilizations are studied using different ways and carriers such as covalent binding, encapsulation, adsorption and entrapment, as immobilization with polylactic acid an eco-friendly (Calzoni *et al.*, 2021), Celite 545 (de Oliveira *et al.*, 2021), Materium 540 (MAT 540) (de Oliveira *et al.*, 2020), as-spun nanofibrils (Guler *et al.*, 2020), Ca-alginate (Qamar *et al.*, 2020), Eupergit CM (Aslan *et al.*, 2018), cellulose monoacetate/chitosan (Demirkan *et al.*, 2018), cysteine-functionalized MNPs (Masi *et al.*, 2017), hollow core-mesoporous shell silica nanospheres (Ibrahim *et al.*, 2016) and sodium alginate beads (Geethanjali and Subash 2013). Thus, this

study aims to partial purification of protease from Prickly lettuce leaves using ammonium sulfate, immobilization bentonite, characterization and application as antimicrobial against the growth of some food spoilage bacteria as a lone or synergistic with Welsh onion *Allium fistulosum* L.

MATERIALS AND METHODS

Place of work

This study was conducted in the laboratories of the Department of Product Evaluation and Service Performance at the Market Research and Consumer Protection Center, University of Baghdad, Baghdad, Iraq and the Department of Science, College of Basic Education at Mustansiriyah University, Baghdad, Iraq.

Research period

The study was conducted from January 10, 2023, to December 10, 2023.

Crude protease preparation

Protease was extracted from leaves of Prickly lettuce (*Lactuca serriola* L.) using acetone powder. $(\text{NH}_4)_2\text{SO}_4$ at 40-80% saturation was used for the concentration of crude extract. The precipitate was dialyzed against deionized distilled water through (Dialysis membrane Spectra/Por® 7 MWCO 10000 Da) for 24 h at 4°C, then concentrated by freeze dryer (Al-Soufi, 2013).

Protease proteolytic activity

The proteolytic activity of free protease (FP) and immobilized protease (IP) was estimated according to the method of Robinson (1975) by mixing 0.3 mL of FP and 0.3 g of IP with 0.7 mL of 0.1M sodium phosphate buffer activation solution pH (7.2) containing 0.1 M of cysteine and 0.001 M of EDTA, then heated for 5 min at 35°C. Then, 1 mL 0.1 M sodium phosphate buffer solution pH (7.2) containing 1% casein was heated for 5 min at 35°C. Then, the reaction was stopped by adding 3 mL of 5% $\text{Cl}_3\text{CCO}_2\text{H}$ and filtered after 30 min through filter paper (Whatman No. 3), the absorbance of filtrates was measured at 280 nm. Control was prepared by adding $\text{Cl}_3\text{CCO}_2\text{H}$ at zero time of reaction. One unit of the enzyme is defined as the protease activity that changed by 1.0 unit of absorbance min^{-1} under the experiment conditions.

Estimation of protein

Protein was estimated using Bradford's method (1976).

Activation of clay

Clay (bentonite) was obtained from Baghdad's local markets. The activation assay was done by adding 10% 3-APTES solution in acetone to clay (v w^{-1}) and stirring at 25°C for 1 h. After that, filtrating and washing using acetone consecutively three times and then drying by oven at 80°C; the dried bentonite was treated with 10% aqueous glutaraldehyde solution (w v^{-1}) for 60 min. This was followed by filtrating and washing using 0.1 mM of sodium phosphate buffer solution pH (7.2) consecutively three times, then stored in the same

buffer solution at 4°C until using in the immobilization of enzyme according to the method of Alsoufi (2021). The pH modification for the buffer solution was (7.2) instead of (6) to suit the enzyme activity and experimental conditions.

Immobilization of protease

Protease was immobilized by mixing 10 mL of the enzyme (10 mg mL^{-1}) with 10 g of activated clay and slowly stirring (avoid foaming) for 2 h at 4°C, following that, adding 1% of glutaraldehyde in 0.1 mM sodium phosphate buffer solution pH (7.2) and stirrer for 1h at 4°C. The storage was in the refrigerator until use, according to Alsoufi (2019), with the modification of buffer solution to be 0.1 mM sodium phosphate buffer solution, pH (7.2) instead of 50 mM Tris-HCl buffer solution, pH (7).

Immobilization efficiency

The immobilization efficiency (IE) of protease was calculated using Eq. (1) by Alsoufi (2021).

$$\text{IE}(\%) = \frac{\text{IM}}{\text{FA}} \times 100$$

Where IM= Specific activity (U mg^{-1}) of immobilized protease; FA= Specific activity (U mg^{-1}) of free protease.

Optimum activity and stability of pH and temperature

The effect of optimum relative activity (%) for pH and temperature on the FP and IP was assayed using 1% casein in 50mM buffers solutions pH (5-10), sodium acetate (5-6.5), sodium phosphate (7-7.5), Tris-HCl (8-9.5) and Glycine-NaOH (10) for pH and (30-80°C) for temperature (Geethanjali and Subash, 2013). In contrast, the relative stability (%) for pH and temperature was estimated after incubating the enzyme for 30 min (Al-Soufi, 2013).

Storage and reuse

The effect of storage and reuse of protease was estimated according to the method of Alsoufi (2019) by storing FP and IP in optimum pH of stability for 30 d at 4°C and studying the reuse of IP for 30 continued reuse.

Extraction and detection of phytochemicals from welsh onion

The phytochemicals (glycosides, alkaloids, terpenes, saponins, tannins, coumarins and flavonoids) of Welsh onion *Allium fistulosum* L. (WO) were extracted and detected according to the method of Alsoufi and Aziz (2022) using hot aqueous extraction through adding 50 g of chopped onion to 100 mL of deionized distilled water and put the mixture in the water bath at 100°C for 1 h. Then, it was left to cool at room temperature and filtered through filter paper (Whatman No.1). The filtrate was used to detect phytochemicals, then dried by lifolyzer and kept at 4°C until use.

Antimicrobial activity

Antimicrobial activity of FP and IP were studied against *S. aureus* and *E. coli* that were obtained from the Department

of Science, College of Basic Education, Mustansiriyah University, Baghdad, Iraq, through activation of bacterial strains by incubation under sterilized conditions in 20 mL of nutrient broth at for 24 h at 37°C. Then, spread 100 µL on nutrient agar and incubate for 24 h at 37°C. An isolated colony from each strain was placed in physiology serum (deionized distilled water and 0.9% sodium chloride) until they reached 0.5 turbidity of McFarland (10^6 CFU mL⁻¹). The antimicrobial activity was estimated by adding 1 mL of 25, 50, 75 and 100 mg of FP, IP, or WO to 40 mL of nutrient broth. Adding 1 mL of bacterial culture medium to each type of them and incubating the mixture at 37°C for 24 h. Absorbance was measured by UV-Vis spectrophotometer at 610 nm in 2 h time intervals for FP and IP according to the method by Bayazidi *et al.* (2018). After 24 h for WO and synergistic mix, the synergistic antimicrobial activity was distributed (Table 1) according to the method of Alsoufi and Aziz (2022).

The changes in absorbance (%) of all treatments were calculated using Eq. (2) (Alsoufi and Aziz, 2022).

$$\text{Change in absorbance (\%)} = \frac{(a-b) - (c-b)}{(a-c)} \times 100$$

Where:

a= Absorbency of control after incubation at 37°C for 24 h.

b= Absorbency of treatment at zero time of incubation at 37°C.

c= Absorbency of treatment after incubation at 37°C for 24 h.

Statistical analysis

SAS (Statistical Analysis System) program version 9.6th ed., was used to detect the difference factors in the experiments of this study using LSD (Least significant difference) test (Analysis of Variation-ANOVA) to compare between means in results (SAS, 2018).

RESULTS AND DISCUSSION

Immobilization efficiency

The efficiency of protease immobilization was 78% of the original amount of enzyme used. In this context, the immobilization efficiency of protease with different sources was 50-55% with as-spun nanofibrils (Guler *et al.*, 2020), 68% on polylactic acid an eco-friendly (Calzoni *et al.*, 2021), 45% with sodium alginate beads (Geethanjali and Subash, 2013), 71% with Eupergit CM (Aslan *et al.*, 2018), 83% on cellulose monoacetate/chitosan (Demirkan *et al.*, 2018)

and 75.6% onto hollow core-mesoporous shell silica nanospheres (Ibrahim *et al.*, 2016).

The main aim of immobilization was to get an enzyme kit with high activity, stability, reusable and low cost (Alsoufi, 2021), which was achieved by binding the highest amount of enzyme with inert materials unescorted by any losses of activity and stability such as polymers and inorganic materials (Alsoufi, 2019) that provided non-effect of catalytic property in the active site due the binding with immobilization materials (Masi *et al.*, 2017).

Effect of optimum pH and temperature

The optimum activity pH for FP and IP was (8.5) (Fig 1). The stability for 30 min of FP (the losses less than 10%) was at the pH range of (7.5-9) with loss of 21 and 14% of its original activity at pH (5 and 10), respectively. The stable of IP was at the pH range (5-10) with a loss of 15 and 7% of its original activity at pH (5 and 10), respectively (Fig 2). The optimum activity temperature for FP and IP was 60°C (Fig 3). The FP was stable for 30 min at 55°C, while IP was stable for 30 min at 60°C (without any losses of its activity) (Fig 4).

Many research studies refer to variances in the optimum temperature of protease from different sources. On this basis, Calzoni *et al.* (2021) observed that the optimum conditions of IP on eco-friendly polylactic acid were at a temperature of 55°C and a pH of (8.6). At the same time, de Oliveira *et al.* (2021) found that the highest activity of temperatures and pH for IP from *Rhodotorula oryicola* on Celite 545 were 60°C and (6.5), respectively. de Oliveira *et al.* (2020) noticed the same results for IP from *Moorella speciosa* on Materium 540 (MAT 540). Qamar *et al.* (2020) showed that the optimum pH of protease from *Bacillus brevis* was (8 and 10) for FP and IP on Ca-alginate, respectively and the optimum temperature was at 45 and 65°C, respectively. Aslan *et al.* (2018) noted that the optimum pH and temperature for IP from *Bacillus licheniformis* on Eupergit CM were (7-8) and 70°C, respectively. Similarly, Masi *et al.* (2017) observed that the optimum pH of FP and IP from (*P. aeruginosa* and *Enterococcus hirae*) with cysteine-functionalized MNPs was (9) and the optimum temperature was 60°C for both types of enzyme, with recorded more stability to IP than FP during incubation for 5 h at 60°C and pH (9). Geethanjali and Subash (2013) reported that the optimum pH and temperature of protease from *Labeo rohita* visceral were

Table 1: Distribution rate (%) of synergistic antibacterial activity of immobilized protease from Prickly lettuce (*Lactuca serriola* L.) leaves on bentonite and dried extract of Welsh onion (*Allium fistulosum* L.).

Treatment no.	Antibacterial solution	Rate (%)
1	Immobilized protease (IP)	100
2	Welsh onion (WO)	100
3	Immobilized protease (IP) + Welsh onion (WO)	75 + 25
4	Immobilized protease (IP) + Welsh onion (WO)	50 + 50
5	Immobilized protease (IP) + Welsh onion (WO)	25 + 75
6 (Control)	Distilled water	100

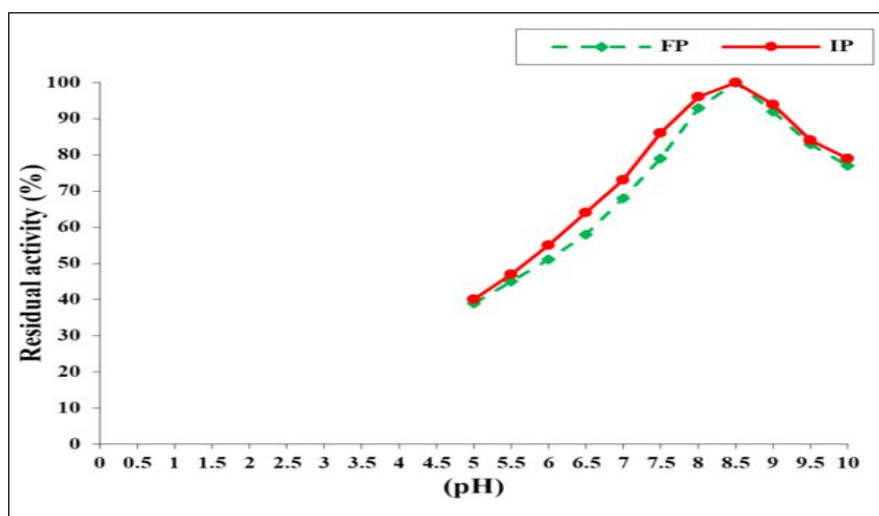


Fig 1: Effect of optimum pH on activity for free (FP) and immobilized (IP) protease from Prickly lettuce leaves on bentonite.

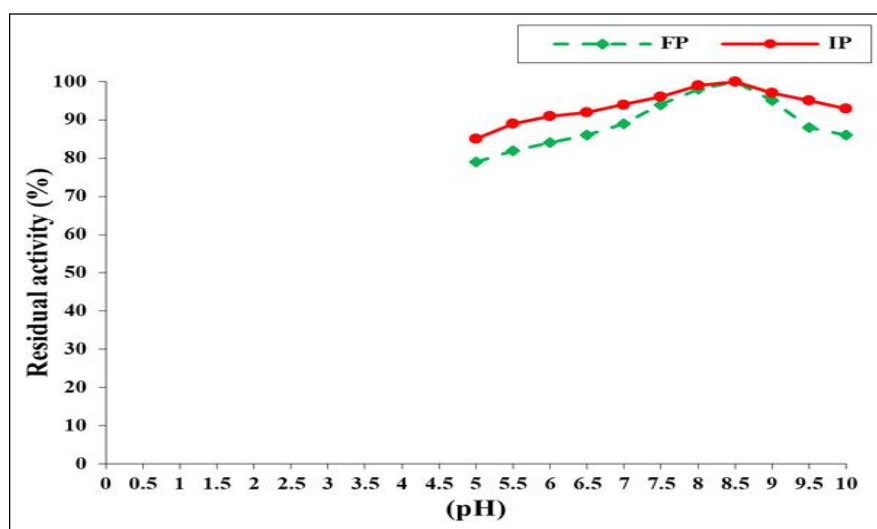


Fig 2: Effect of optimum pH on stability for free (FP) and immobilized (IP) protease from Prickly lettuce leaves on bentonite.

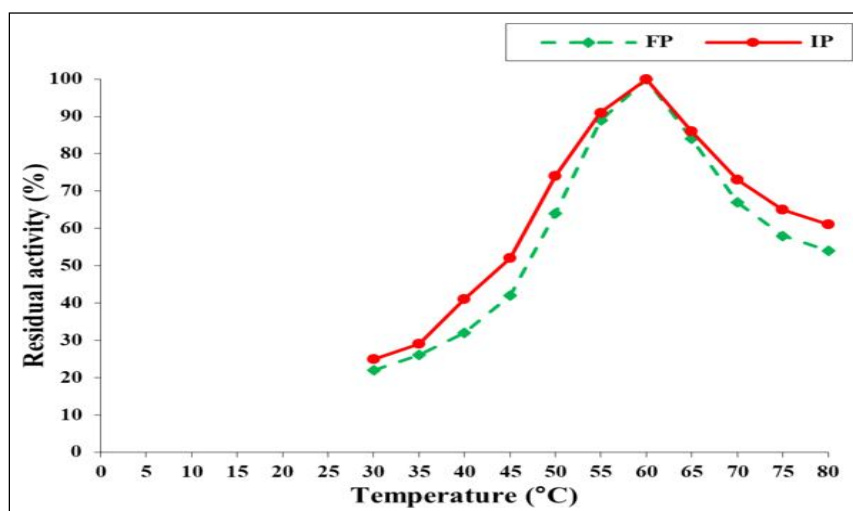


Fig 3: Effect of optimum temperature on activity for free (FP) and immobilized (IP) protease from Prickly lettuce leaves on bentonite.

(9) and 60°C, respectively, for FP and IP with sodium alginate beads, noting that the IP was a high activity of 98% even at 50°C compared to FP.

The pH and temperature values of protease were varied relying on the source, type, pl, molecular weight and the content of carbohydrate and substrate (Al-Soufi, 2013), each enzyme has an optimum value of these parameters and the enzyme can incur ionizations and acquire charges due pH of reaction solutions. Temperature may affect the amino acid bonds, leading to a loss of enzyme activity (de Oliveira *et al.*, 2021). Generally, immobilization increases the stability of the enzyme towards pH and temperature, which were used in the reaction due to strengthening the enzyme's three-dimensional structure via covalent bonds (Aslan *et al.*, 2018; Ibrahim *et al.*, 2016).

Storage and reuse

The IP did not lose activity for 15 d while losing 57% over 30 d of storage at 4°C; the FP was stable for 4 d while losing all activity over 17 d at 4°C (Fig 5). The IP activity was

stable at up to 15 reuses, losing 24% of its initial activity after 30 reuses (Fig 6).

The stability of immobilized enzymes through reuse and storage is an essential requirement for the success of its applications, which reduces the price and increases suitability for commercial applications (Alsoufi and Aziz, 2022; Geethanjali and Subash, 2013). The total enzyme activity of IP on polylactic acid was eco-friendly (4:1) to FP after 100 reuses (Calzoni *et al.*, 2021). The IP from *R. oryzae* on Celite 545 retained up to 73% of activity after 15 reuses (de Oliveira *et al.*, 2021). de Oliveira *et al.* (2020) found that IP from *M. speciosa* on MAT540 retained 60% of the initial catalytic activity after 15 reuses in this context. Qamar *et al.* (2020) showed that the IP from *Bacillus brevis* on Ca-alginate retained more than 80% of original activity up to 8 reuses. Aslan *et al.* (2018) observed that IP from *Bacillus licheniformis* on Eupergit CM has not lost activity through 20 reuses. The IP from *Bacillus* sp. on hollow core-mesoporous shell silica nanospheres retained up to 55.6% of activity after 12 reused (Ibrahim *et al.*, 2016).

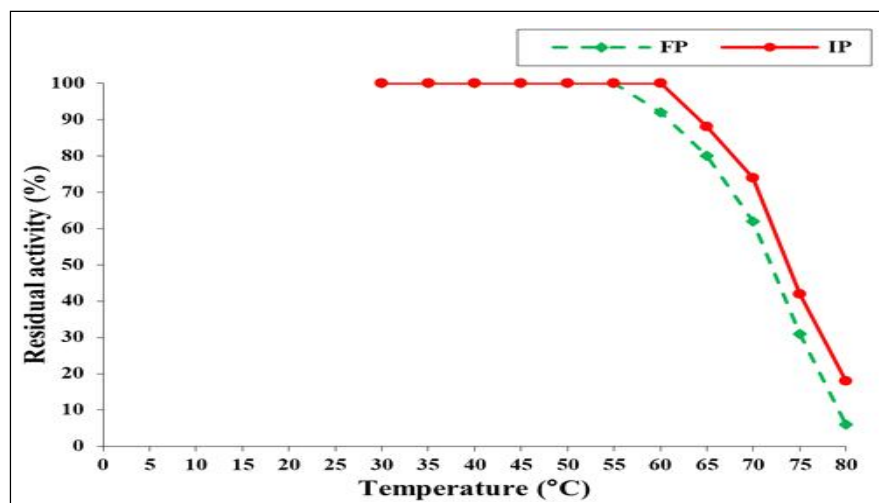


Fig 4: Effect of optimum temperature on stability for free (FP) and immobilized (IP) protease from Prickly lettuce leaves on bentonite.

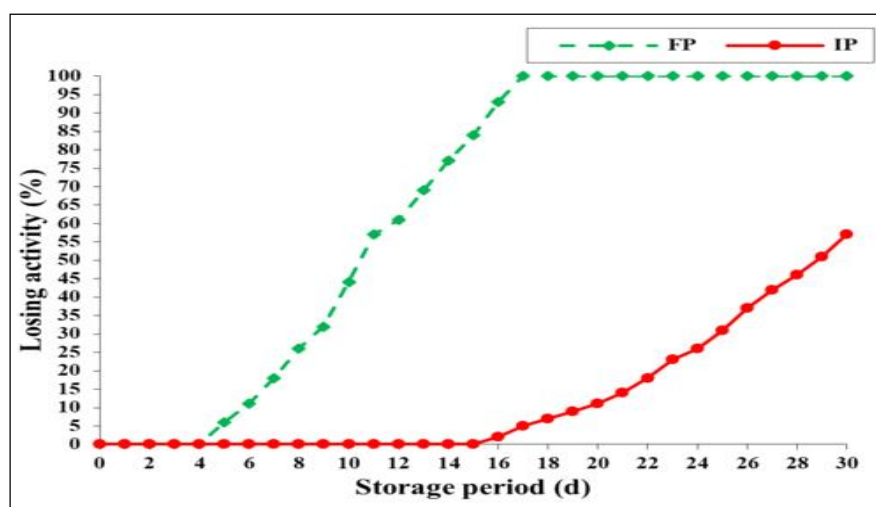


Fig 5: Effect of storage period at 4C on free (FP) and immobilized (IP) protease from Prickly lettuce leaves on bentonite.

Geethanjali and Subash (2013) noted that IP from *Labeo rohita* with sodium alginate beads was more stable at 4°C compared to 25°C during storage for 6 d with near retention of 90% and 20% from original activity, respectively. The losing activity of immobilized enzymes during reuse constitutes a central problem affecting their applications due to the leakage of an enzyme from carrier material used for immobilization. The effect of mechanical forces for centrifugation after all use and cover catalytic site in the enzyme's active site by accumulating substrate oxidation products (Qamar *et al.*, 2020; Alsoufi, 2019; Ibrahim *et al.*, 2016). Therefore, this parameter was used to choose the appropriate application and ensure marketing success (Alsoufi, 2021). Immobilization generally improves enzyme stability, while losing protease activity is attributed to autolysis at a high temperature (Geethanjali and Subash, 2013).

Phytochemicals of welsh onion

The detection of phytochemical substances found in Welsh onion refers to the hot aqueous extract of contain

(glycosides, alkaloids, terpenes, saponins, tannins and flavonoids).

Welsh onion has many pharmacological activities (antimicrobial, antifungal, anti-inflammatory, antioxidant, anticancer) and traditional uses as an herbal medicine (Sung *et al.*, 2018) due to the phytochemical substances found in the plant, such as flavonoids, terpenes, glycosides, saponins, alkaloids and tannins (Alsoufi and Aziz, 2022).

Application

The result showed that the antimicrobial effect of IP was greater than that of FP on *S. aureus* and *E. coli*. The optimum concentration of enzyme used was 100mg over incubation of each type of bacteria for 24 h at 37°C (Fig 7; 8). The antimicrobial effect of hot aqueous extract (WO) on inhibition growth of *E. coli* was more than *S. aureus*; the optimum concentration of extract used was 100 mg over incubation of each type of bacteria for 24 h at 37°C (Fig 9). The synergistic effect from 50 mg of IP and 50 mg of (WO) showed a noticeable increase in inhibition growth of *S. aureus* and *E. coli* (Fig 10). The absorbance (%) at 610

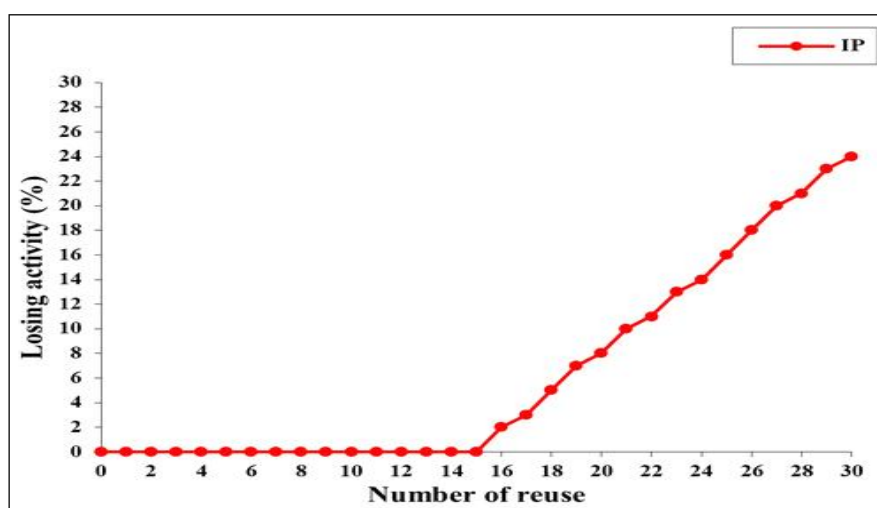


Fig 6: Effect of reuse on immobilized protease (IP) from Prickly lettuce leaves on bentonite.

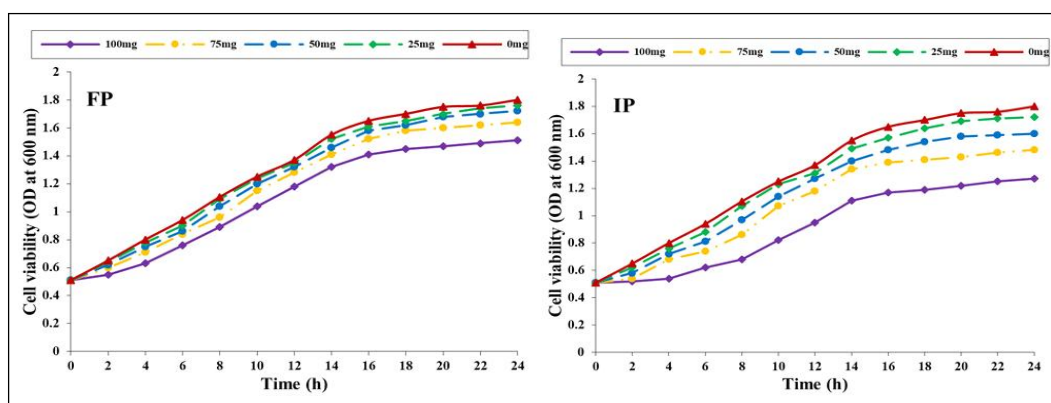


Fig 7: Antimicrobial effect of free (FP) and immobilized (IP) protease from Prickly lettuce leaves with bentonite (mg) on inhibition growth of *Staphylococcus aureus*.

nm for growth of bacteria over 24 h at 37°C of incubation showed significant change ($P \leq 0.01$) compared to the control to be 22.48 ± 1.37 , 45.74 ± 2.06 , 33.33 ± 1.79 , 63.75 ± 3.64 and $82.17 \pm 4.05\%$ for *S. aureus*, while it was 9.52 ± 0.71 , 68.71 ± 3.52 , 45.59 ± 2.75 , 77.55 ± 3.81 and

$92.52 \pm 3.98\%$ for *E. coli* when using 100, 100, 75+25, 25+75, 50+50 mg of IP and (WO), respectively (Table 2).

The achievement of food safety represented the main aim of food production due to consumers' health concerns related to food spoilage and the increasing demand to

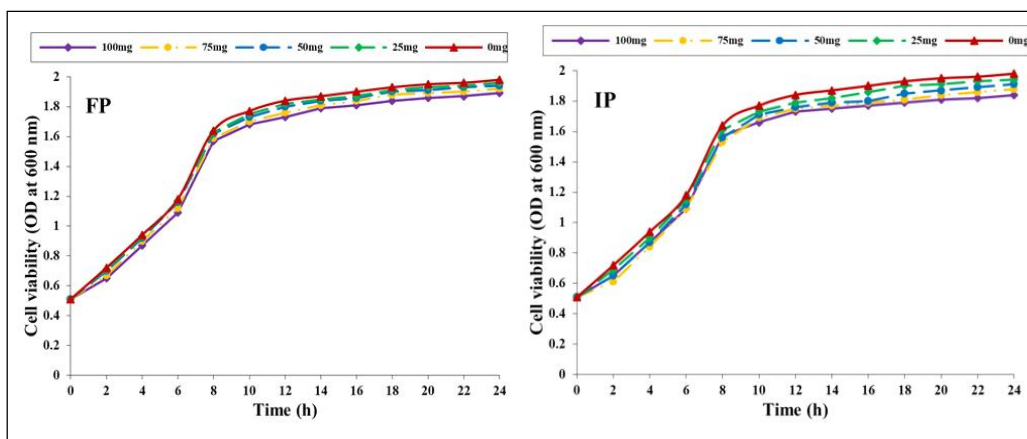


Fig 8: Antimicrobial effect of free (FP) and immobilized (IP) protease from Prickly lettuce leaves with bentonite (mg) on inhibition growth of *Escherichia coli*.

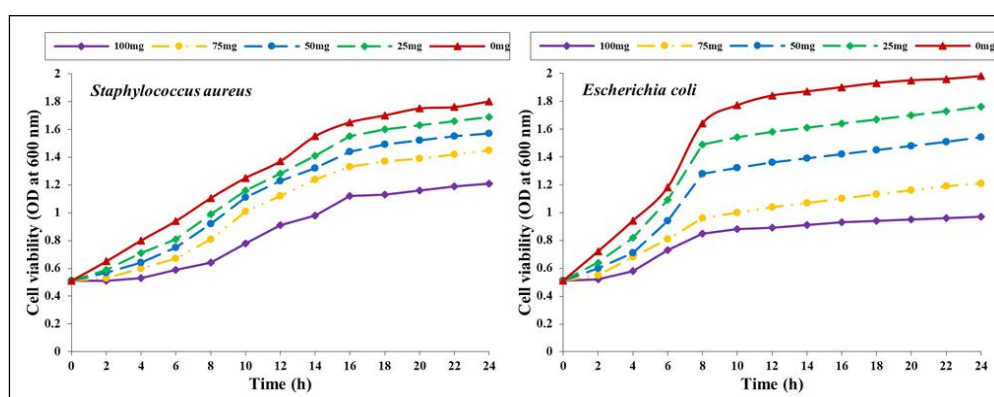


Fig 9: Antimicrobial effect of hot aqueous extract of Welsh onion (mg) on inhibition growth of *Staphylococcus aureus* and *Escherichia coli*.

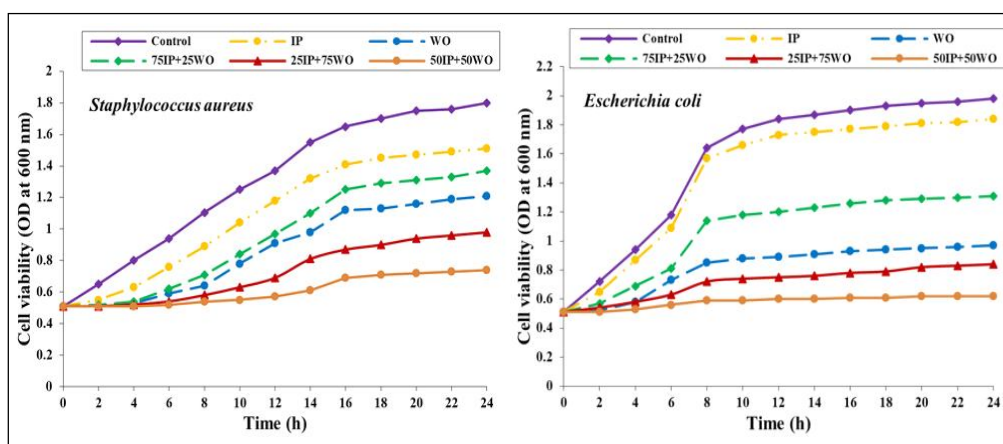


Fig 10: Effects of synergistic antimicrobial solutions of immobilized protease (IP) from Prickly lettuce leaves on bentonite and hot aqueous extract of welsh onion (WO) (mg) on inhibition growth of *Staphylococcus aureus* and *Escherichia coli*.

Table 2: Change in absorbance (%) at 610 nm for *S. aureus* and *E. coli* growth over incubation for 24 h at 37°C through the use of different concentrations of immobilized protease IP and hot aqueous extract of Welsh onion (WO).

Type of treatment	Change in absorbance (%)	
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
Control	0.0±0.0 f	0.0±0.00 d
Immobilized Protease (IP) (100 mg)	22.48±1.37 e	9.52±0.71 d
hot aqueous extract of Welsh onion (WO) (100 mg)	45.74±2.06 c	68.71±3.52 b
Immobilized Protease (IP) (75 mg) + hot aqueous extract of Welsh onion (WO) (25 mg)	33.33±1.79 d	45.59±2.75 c
Immobilized Protease (IP) (25 mg) + hot aqueous extract of Welsh onion (WO) (75 mg)	63.75±3.64 b	77.55±3.81 b
Immobilized Protease (IP) (50 mg) + hot aqueous extract of Welsh onion (WO) (50 mg)	82.17±4.05 a	92.52±3.98 a
LSD (P-value)	8.037 *(0.0001)	11.163 *(0.0001)

This means having the different letters in the same column differed significantly, *(P≤0.01).

enhance the shelf life of food using natural ingredients and preservatives and reduce the chemical materials use (Alsoufi and Aziz, 2022). Therefore, many authors reported that the effectiveness of proteases an antimicrobial and forming of biofilm as alone or synergistic with herbal drugs, phytochemicals and synthetic antibiotics (Salinas Ibáñez *et al.*, 2021) due to the ability of lyses cell walls by cleavage the pentaglycine bridges between the peptidoglycan and reduce the amount of carbohydrate and protein contents in the biofilms formed (Manohar *et al.*, 2015). Such as proteases from Dunal fruit against *Helicobacter pylori* (Salinas Ibáñez *et al.*, 2021), protease from *Xylaria psidii* KT30 against *B. subtilis* and *S. aureus* (Indarmawan *et al.*, 2016), Papain cross-linked polymers against *Acinetobacter* sp. KC119137.1 and *S. aureus* (Manohar *et al.*, 2015), subtilisin against *Pseudomonas fluorescens* (Molobela *et al.*, 2010) and *L. monocytogenes* (Longhi *et al.*, 2010).

CONCLUSION

The study shows the possibility of immobilization of partial purification protease from Prickly lettuce *Lactuca serriola* L. leaves on bentonite with good efficiency of immobilization, storage, reuses and suitable pH and temperature of proteolytic activity of enzyme, for using it as an antimicrobial agent against food spoilage bacteria, particularly *Staphylococcus aureus* and *Escherichia coli*, either alone or in synergistic with extract of Welsh onion *Allium fistulosum* L.

Conflict of interest

The authors declare that there is no conflict of interest regarding this work

REFERENCES

Abdullah, N.H., Kamaruding, N., Eldeen, I.M., Taib, M. and Ismail, N. (2022). Characterization of trypsinlike serine proteases from *Tachypleus tridentatus* amebocyte lysate and its potential for endotoxin detection. Indian Journal of Animal Research. doi: 10.18805/IJAR.BF-1574.

Al-Soufi, M.A. (2013). Partial purification and estimated some characterization of protease from prickly lettuce (*Lactuca serriola* L.) leaves and used it in some applications. Iraqi Journal of Biotechnology. 12(2): 1-18.

Alsoufi, M.A. (2019). Use of immobilized L-arabinose isomerase for production of tagatose. Iraqi Journal of Market Research and Consumer Protection. 11(2): 122-131.

Alsoufi, M.A. (2021). Use of immobilized pectinase for fruit juice clarification. Bioscience Research. 18(2): 1480-1487.

Alsoufi, M.A. and Aziz, R.A. (2022). Use of biopreservation technique to increase shelf life for some types of meat. Bioscience Research. 19(2): 1133-1138.

Aslan, Y., Ömerosmanoğlu, D. and Koç, E.O. (2018). Covalent immobilization of an alkaline protease from *Bacillus licheniformis*. Turkish Journal of Biochemistry. 43(6): 595-604.

Bayazidi, P., Almasi, H. and Asl, A.K. (2018). Immobilization of lysozyme on bacterial cellulose nanofibers: Characteristics, antimicrobial activity and morphological properties. International Journal of Biological Macromolecules. 107(B): 2544-2551.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72(1-2): 248-254.

Calzoni, E., Cesaretti, A., Tacchi, S., Caponi, S., Pellegrino R.M., Luzi, F., Cottone, F., Fioretto, D., Emiliani, C. and Di Michele, A. (2021). Covalent immobilization of proteases on polylactic acid for proteins hydrolysis and waste biomass protein content valorization Catalysts. 11(2): 167.

de Oliveira, J.M., Fernandes, P., Benevides, R.G. and de Assis, S.A. (2021). Production, characterization and immobilization of protease from the yeast *Rhodotorula oryzzicola*. Applied Biochemistry and Biotechnology. 68(5): 1033-1043.

de Oliveira, J.M., Fernandes, P., Benevides, R.G. and de Assis, S.A. (2020). Characterization and immobilization of protease secreted by the fungus *Moorella speciosa*. 3 Biotech. 10: 419.

Demirkan, E., Avci, T. and Aykut, Y. (2018). Protease immobilization on cellulose monoacetate/ chitosan-blended nanofibers. Journal of Industrial Textiles. 47(8): 2092-2111.

- Dinani, O.P., Tyagi, P.K., Mandal, A.B., Tyagi, P.K. and Bhanja, S.K. (2021). Cost economics of feeding rice gluten meal and soybean meal through dietary addition of enzymes in broilers. *Agricultural Science Digest*. 41(4): 578-583. doi: 10.18805/ag.D-5233.
- Fguiri, I., Sboui, A., Atigui, M., Arroum, S., Ayeb, N., Dbara, M., Hammadi, M. and Khorchani, T. (2023). Camel milk curd properties: Application of a kiwi juice as a coagulant. *Asian Journal of Dairy and Food Research*. 42(1): 14-19. doi: 10.18805/ajdfr.DRF-275.
- Geethanjali, S. and Subash, A. (2013). Optimization and immobilization of purified *Labeo rohita* visceral protease by entrapment method. *Enzyme Research*. Article ID 874050.
- Guler, B.E., Demirkan, E. and Sevgi, T. (2020). Immobilization of *Bacillus subtilis* E6-5 protease and commercial protease in nanofibrils containing different amino acids. *Trakya University Journal of Natural Sciences*. 21(1): 39-45.
- Ibrahim, A.S.S., Al-Salamah, A.A., El-Toni, A.M., Almaary, K.S., El-Tayeb, M.A., Elbadawi, Y.B. and Antranikian, G. (2016). Enhancement of alkaline protease activity and stability via covalent immobilization onto hollow core-mesoporous shell silica nanospheres. *International Journal of Molecular Sciences*. 17(2): 184.
- Indarmawan, T., Mustopa, A.Z., Budiarto, B.R. and Tarman, K. (2016). Antibacterial activity of extracellular protease isolated from an algicolous fungus *Xylaria psidii* KT30 against gram-positive bacteria. *HAYATI Journal of Biosciences*. 23(2): 73-78.
- Longhi, C., Scoarughi, G.L., Poggiali, F., Cellini, A., Carpentieri, A., Seganti, L., Pucci, P., Amoresano, A., Cocconcelli, P.S., Artini, M., Costerton, J.W. and Selan, L. (2010). Protease treatment affects both invasion ability and biofilm formation in *Listeria monocytogenes*. *Microbial Pathogenesis*. 45(1): 45-52.
- Manohar, C.M., Prabhawathi, V., Sivakumar, P.M. and Doble, M. (2015). Design of a papain immobilized antimicrobial food package with curcumin as a crosslinker. *PLOS ONE*. 10(4): e0121665.
- Masi, C., Chandramohan, C. and Ahmed, M. F. (2017). Immobilization of the magnetic nanoparticles with alkaline protease enzyme produced by *Enterococcus hirae* and *Pseudomonas aeruginosa* isolated from dairy effluents. *Brazilian Archives of Biology and Technology*. 60: e17160572.
- Mirza, A., Srujana, A. and Singh, J. (2023). Study on measuring proteolytic activity and phyto chemical analysis of fruit latex and leaves extract of papaya (*Carica papaya* L.) Cv. CO2. *Indian Journal of Agricultural Research*. doi: 10.18805/IJARe.A-6096.
- Molobela, P., Cloete, T.E. and Beukes, M. (2010). Protease and amylase enzymes for biofilm removal and degradation of extracellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* bacteria. *African Journal of Microbiology Research*. 4(14): 1515-1524.
- Qamar, S.A., Asgher, M. and Bilal, M. (2020). Immobilization of alkaline protease from *Bacillus brevis* using Ca-alginate entrapment strategy for improved catalytic stability, silver recovery and dehairing potentialities. *Catalysis Letters*. 150: 3572-3583.
- Robinson, G.W. (1975). Isolation and characterization of papaya peptidase A from commercial chymopapain. *Biochemistry*. 14(16): 3695-3700.
- Salinas Ibáñez, Á.G., Vallés, D., Adaro, M., Barberis, S. and Vega, A.E. (2021). Antimicrobial effect of a proteolytic enzyme from the fruits of *Solanum granulosum-leprosum* (Dunal) against *Helicobacter pylori*. *Frontiers in Nutrition*. 8: 69995-52021.
- SAS. (2018). Statistical Analysis System, User's Guide. Statistical," Version 9.6th ed. SAS. Inst. Inc. Cary. N.C. 2018.
- Sung, Y.Y., Kim, D.S., Kim, S.H. and Kim, H.K. (2018). Aqueous and ethanolic extracts of welsh onion, *Allium fistulosum*, attenuate high-fat diet-induced obesity. *BMC Complementary and Alternative Medicine*. 18(1): 105.