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Biochemistry

Improvement of Extracellular Chitinase Enzyme Produced by *Citrobacter freundii* Through Nano Preparation Technology

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ABSTRACT

A chitinolytic enzyme has been isolated from the marine bacterium *Citrobacter freundii* after a bioremediation process on fish wastes. The microbe was grown on nutrient broth medium supplemented with 1, 5, and 10% wastes. Enzyme production reached 70 u/ml after 9 days of incubation. At 40°C, the activity was raised to almost three times making the whole process a thermophilic bioremediation. The extracellular enzyme showed a maximum activity when the pH value was slightly alkaline (pH 8). Salinity greatly affects the enzymatic activity which was at least doubled at different pH. Maximum extracellular chitinase activity was obtained when 0.5% fish waste was used as the microbiological medium for the bioremediation process. The extracellular enzyme was purified by salting out using ammonium sulfate at 65% concentration followed by fractionation on Sephadex G-100 column. The gel filtration step resulted in more than 85 purification fold. The enzyme retained more than 70% of its activity at 60°C and pH 8.0 making it a very good candidate for almost all industrial processes. The activity of purified chitinase was greatly affected after enzyme nanoparticle formation on ferric oxide. Enzyme immobilization kept more than 90% of its activity for more than 6 weeks at 35°C. Moreover, 55°C was the optimum temperature for both purified and immobilized enzyme samples. The activity of enzyme-nanoparticles preparation reached more than double the activity of the purified enzyme alone at the same temperature.

Keywords: *Citrobacter freundii,* Chitinase, enzyme nanoparticles, enzyme purification, next-generation identification, Gel filtration chromatography.

INTRODUCTION

Chitin, a widespread natural polymer, is a vital component of mammalian cell structures. It is commonly found in fungal cell walls, insect peritrophic membranes, and nematode egg shells (Qin et al., 2016). This macromolecule primarily protects organs from external threats (Stoykov et al., 2015). Chitinolytic enzymes, capable of degrading chitin, have been isolated

from a diverse range of sources, including plants, fungi, yeasts, bacteria, insects, and even vertebrates (Bhattacharya et al., 2007). Marine waste, particularly crustacean shells, is recognized as the primary source of chitin (Farag et al., 2016). Due to chitin's insolubility in water and common organic solvents, researchers have developed various methods employing mineral acid solutions, hexafluoroacetone, N, N dimethylacetamide/LiCl, and hexafluoro-2-propanol to enhance its solubility. Different types of chitins can serve as substrates for measuring chitinase activity (Stoykov et al., 2015).

Chitinases break down chitin by hydrolyzing its glycosidic bonds (Singh et al., 2021; Abulikemu et al., 2021). They are categorized into endochitinases and exochitinases, based on their mode of action. Endochitinases randomly cleave internal sites of the chitin polymer, producing smaller, multi-subunit glucosamine residues (Rathore et al., 2015). Chitinases (EC 3.2.1.14), endo-chitodextrinases (EC 3.2.1.202), and chitosanases (EC 3.2.1.132) are all endo-acting enzymes. Chitinases randomly cleave the chitin chain, producing water-soluble oligomers of N-acetylglucosamine. Endo-chitodextrinase hydrolyzes chitodextrins to release N, N′-diacetylchitobiose, with small amounts of N, N′, N″-triacetylchitotriose (Poria et al., 2021).

Enzymes employed in biocatalysis are typically isolated from microbial cells cultured in specific media. While organic solvents can be used for enzyme purification, salting out with ammonium sulfate remains a widely adopted method due to its numerous benefits (Wingfield P., 2001). This technique exploits the differential precipitation of proteins at varying ammonium sulfate concentrations. By adding different amounts of ammonium sulfate to cell extracts and centrifuging to remove precipitated proteins, enzyme activity can be assessed in the remaining supernatant. Chromatography techniques, such as gel filtration, adsorption chromatography, and affinity columns, are also utilized for enzyme purification. These procedures are crucial for obtaining pure proteins, which are essential for advancing biocatalysis research and commercial applications. In recent years, affinity chromatography techniques, especially those involving recombinant proteins fused with specific tags, have largely replaced traditional methods for their efficiency in single-step purification (Westphal & van Berkel, 2021).

Bacterial chitinases have diverse commercial applications. For instance, they are used to create fungal protoplasts and produce chitin oligomers with antibacterial and antifungal properties (Bhattacharya et al., 2007). Fungi, a major cause of plant diseases, account for over 70% of crop losses. Chitinase enzymes are effective in controlling fungal pathogens (Gomaa, 2021). Recombinant chitinases can convert chitinous biomass into simpler components, reducing water pollution.

Additionally, chitinases are being explored for diagnosing invasive fungal infections in humans (Rathore & Gupta, 2015). The production of chitinases has been used as a criterion for selecting potential insect-control agents (Nagpure et al., 2013). Recently, chitinases have also shown anticancer effects, with studies demonstrating their ability to destroy cancer cells in mice (Pan et al., 2005). Early research suggests that acidic mammalian chitinase (AMCase)

is involved in asthma (Beier and Bertilsson 2013; Stoykov et al., 2015). Chitin oligosaccharides (COS) hold significant potential in food bio-preservation, acting as antimicrobial agents against various food pathogens (Gomaa, 2021).

Enzyme biotechnology has expanded its research focus to enhance enzyme characteristics (Pundir, C.S., 2015). Enzyme nanotechnology is one such research area, as nanotechnology can reduce costs in industries that address pollution through eco-friendly nanomaterial production. The superior physical, chemical, and mechanical properties of nanomaterials make them attractive (Nikalje, 2015; Loureiro et.al., 2016). The use of microorganisms to produce nanoparticles further promotes green biotechnology as a sustainable and cost-effective approach to nanotechnology (Mandeep & Shukla, 2020).

MATERIALS AND METHODS

Microbial isolation and identification

Samples were taken from fish wastes in fresh and salt waters, sites of (Alexandria government). Samples were diluted using sterile saline tubes concentration (9 g/L). Hyper chitinase isolate was chosen for the rest of study. Microbial identification was found to be *Citrobacter freundii*. Analysis was done by The MALDI Biotyper® next-generation microbial identification system (Sherry et al., 2018)

Determination of colony-forming units

Determination of colony forming unit per ml for isolated bacteria strain was done by using serial dilution method. Inoculated petri plates were incubated for 48h at 30^{o}C (Sieuwerts et al., 2008).

Microbiological media

Nutrient broth medium contained 5 g sodium chloride, 5 g peptone, and 3 g beaf extract. All contents were brought up to one liter with distilled water. pH was adjusted to 7.0 with diluted NaOH. Nutrient agar medium was Nutrient broth medium that contained 20 g agar per liter (Difco, 1974). Chitin agar medium contained 10 g colloidal chitin, 5 g yeast extract, 0.5 g magnesium sulfate, 2 g sodium nitrate, 0.5 g potassium chloride, 1 g dipotassium hydrogen phosphate, and 20 g agar. All contents were brought up to one liter with distilled water. pH was adjusted to 7.0 with diluted NaOH (Sherief et al., 1991). Fish waste medium was nutrient broth medium supplemented with different concentrations of treated fish waste.

Treatment of fish waste

Fish waste collected from sea fish samples were washed with distilled water. Clean samples were dried at 50^{o}C overnight. Samples were then ground with a bench blinder. Powdered fish waste samples were stored at room temperature for further research steps.

Effect of physical and biochemical parameters on bacterial growth

The effect of pH on bacterial growth and chitinase production was studied using buffered nutrient broth. The following buffer systems were used, Citrate phosphate buffer pH 5.5, Phosphate buffer pH 7.0, pH 8.0, Tris- HCl buffer pH 9.0.

To determine the optimum temperature for growth and enzymatic activity by the selected bacterial isolate, the isolate was grown on nutrient broth medium for the indicated time with shaking at 180 rpm. The medium was adjusted at pH 8.0, and

growth was carried out at different temperatures (20-55^{o}C).

To determine the optimum waste concentration for growth and enzymatic activity by the selected bacterial isolate, the isolate was grown on a modified basal minimum broth medium Basal medium II that contained 0.5 g NH_4Cl, 15 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, 0.1 g $MgCl_2.6H_2O$, and 0.1 g yeast extract per liter (Williams et al., 1990). The medium was supplemented with different concentrations of dried fish waste. Microbial growth was carried out for three days at pH 8.0 and 40^{o}C with shaking at 180 rpm.

Production of chitinase enzyme

Preparation of colloidal chitin was performed from commercial chitin (C9752, Sigma-Aldrich Co, USA). Five grams of chitin powder was added to 60ml of concentrated HCl and left in the refrigerator (at 4^{o}C) overnight with vigorous stirring. The mixture was brought to two liters of ice-cold ethyl alcohol (95%) with rapid stirring and kept overnight at room temperature (25^{o}C). The precipitate was collected by centrifugation at 6000 rpm (4^{o}C) for 25 min. The precipitate was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0) and then stored at 4^{o}C for further applications (Wen et al., 2002; Kuzu et al., 2012)

Determination of total protein

Total protein was determined calorimetrically by the method of Lowery (Lowery et al, 1951).

Assay of Chitinase enzyme activity

Clear zone detection

The ability of the bacterial isolate to produce chitinase enzyme was investigated by putting a disk of bacterial isolate on the surface of the chitinase-detection agar medium. Chitinase activity was determined visually after 3 days at 40^{o}C. The clear zone formed surrounding the colonies on the chitin agar medium plate was measured (Huang et al, 1996).

Quantitative assay of chitinase activity

DNS (dinitrosalicylic acid) method is a common technique used to measure chitinase activity by measuring the amount of reducing sugars (primarily N-acetylglucosamine) released from chitin hydrolysis. Chitin suspension was prepared in Tris-HCl buffer pH 7. A proper amount of chitinase enzyme was added to the chitin suspension. The reaction mixture was incubated at 55°C for 60 minutes. One ml Trichloroacetic acid (TCA) was added to stop the enzymatic reaction. The mixture was centrifuged at 4000 for 10 minutes. DNS reagent was added to the reaction mixture when it was boiled for 10 minutes. The reaction was left to cool at room temperature. The absorbance of the colored solution was measured at usually 530 nm. The absorbance was compared to a standard curve prepared using known concentrations of N-acetylglucosamine.

One unit of Chitinase activity was defined as the amount of enzyme that released 1 µmol of GlcNAc from colloidal chitin per minute (Miller, 1959).

Purification of extracellular chitinase enzyme

Precipitation of extracellular cellulase by $(NH_4)_2SO_4$

The extracellular chitinase produced by *Citrobacter freundii* was precipitated separately by ammonium sulfate to choose a convenient method to purify

the enzyme. A hundred ml fermentation medium was inoculated with recently activated bacterial cells. Culture was allowed to grow for nine days at 40°C with shaking after which it was centrifuged at 6,000 rpm for 30 minutes. One ml sample was taken from crude enzyme solution to determine the protein content and the chitinase activity. The mixture was allowed to stand at -20°C for 1 hour. The crude enzyme sample was precipitated with solid ammonium sulfate (65% saturation) in an ice bath then it was allowed to stand for 1 hour at 4°C. The above mixture was centrifuged at 6,000 rpm for 30 minutes. Pellets were dissolved in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.0 Enzyme samples were determined as above. (Eldourghamy et al., 2016)

Gel purification chromatography

The extracellular chitinase produced by *Citrobacter freundii* was applied to a Sephadex G-100 size exclusion column. Elution was carried out with 0.1 M Tris-HCl buffer at a flow rate of 0.33 ml per minute. Active fractions were collected to be tested for their enzyme activity. All purification steps were carried out in a cold box.

Nanoparticles Technology

Fe_2O_3 nanoparticles obtained from Central Laboratory for Elemental & Isotopic Analysis, Nuclear Research Centre, Atomic Energy Authority, Cairo, Egypt. Characterization of Fe_2O_3 NPs was carried out by transmission electron microscope (TEM) micrograph and frequency size distribution. TEM images were obtained (using aJEOL-JEM-2100 at EM Unit, Mansoura University, Egypt) at 200 kV (Fig. 1). The following samples were prepared by dispersing drops of the colloid on a copper grid, which was then covered with a carbon film, and the solvent was evaporated. NPs suspensions were prepared at a concentration of 200 mg per liter for measurement of hydrodynamic size (Amrut et al., 2010)

Preparation of Enzyme nanoparticles

Six mg of ferric oxide (Fe_2O_3) nanoparticles, 0.8 ml phosphate buffer pH 7, and 10µl glutaraldehyde were dissolved in 3.54 enzyme solution, the solution was stirred for 1 hr and kept in the refrigerator for further studies (Modenez et al., 2018). Tubes with purified enzyme and purified enzyme-NPs were kept at 4°C for further investigation.

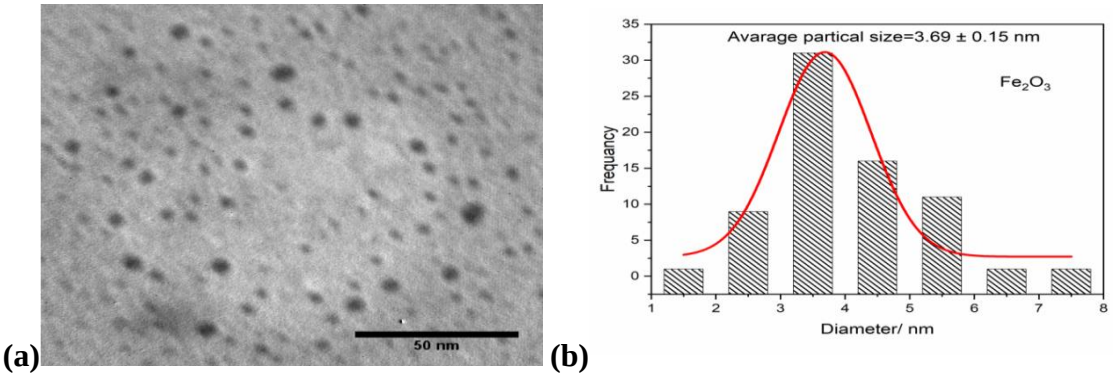


Fig. 1. Characterization of Fe_2O_3 -NPs. (a): (TEM) micrograph and (b): (F) frequency size distribution by TEM.

RESULTS AND DISCUSSION

Screening for chitinase hyper-producing microbes

Several samples were taken from different marine sites (Alexandria Governorate) to select hyperproducers for chitinase production. Sites included sea samples as well as fisheries samples. The most producing microbe was purified for the rest of study. The selected microbe was identified as *Citrobacter freundii* (Table 1). Analysis was done by The MALDI Biotyper® which is an automated next-generation microbial identification system based on matrix-assisted laser desorption/ionization time-of-flight (Sherry et al., 2018).

Table 1. Analysis of isolated bacterial sample by The MALDI Biotyper®, microbial identification system based on matrix-assisted laser desorption/ionization time-of-flight.

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	Citrobacter freundii DSM 30039T HAM	2.24	546
2 (+++)	Citrobacter braakii 9314_2 CHB	2.24	57706
3 (+++)	Citrobacter freundii 13158_2 CHB	2.24	546
4 (+++)	Citrobacter freundii 22054_1 CHB	2.22	546
5 (+++)	Citrobacter braakii 20663_2 CHB	2.21	57706
6 (+++)	Citrobacter freundii DSM 15979 DSM	2.19	546
7 (+++)	Citrobacter youngae DSM 17578T HAM	2.15	133448
8 (+++)	Citrobacter freundii DSM 30039T DSM	2.09	546
9 (+++)	Citrobacter murliniae DSM 13695T HAM	2.06	67829
10 (+++)	Citrobacter freundii LMG 21265 LMG	2.05	546

Bioremediation process

Bioremediation of fish wastes using bacteria is a sustainable and environmentally friendly approach to managing organic pollutants (Kumari et al., 2024). Bacteria possess the metabolic capacity to degrade various components of fish waste, including proteins, lipids, and carbohydrates, into less harmful or harmless compounds. This process reduces the organic load and nutrient pollution associated with fish waste disposal, reducing negative impacts on aquatic ecosystems. Furthermore, bioremediation can generate valuable byproducts such as enzymes, biofuels, and organic fertilizers, enhancing the economic viability of fish waste management.

Citrobacter freundii was grown on nutrient broth medium supplemented with different concentrations (1-10%) of fish wastes for production of extracellular chitinase. Bioremediation process was allowed to stand for 2 weeks to maximize waste utilization.

Enzyme production reached its maximum level after 9 days of incubation. To optimize the whole bioremediation process, different environmental parameters were tested during bacterial growth using the mentioned microbial medium.

Effect of temperature on bacterial growth and enzyme production

Citrobacter freundii was grown on nutrient broth medium supplemented with 0.5% fish wastes. Split samples of the microbe were allowed to grow for 11 days at 30^{o}C and 40^{o}C with shaking (180 rpm). Enzyme production reached almost 12 units per ml after 8 days of incubation at 30^{o}C. Moreover, the activity reached more than 31 units per ml after growing the microbe on the same medium for 9 days at 40^{o}C (table 2) and (figure 2). Obviously, the temperature raised the activity almost three times, making the whole process a thermophilic bioremediation. Lower levels of enzyme production were obtained when the microbe was grown on temperature below 30^{o}C and above 40^{o}C (results are not shown). This is obviously because microbial growth was far from the microbe optimum temperature range of growth.

Table 2. *Citrobacter freundii* chitinase activity (U/ml) after growth on different temperatures

Incubation time (day)	**U/ml at:**	
	30°C	**40°C**
1	2.35	17.4
2	3.21	19.3
3	4.11	21.5
4	4.98	22.7
5	6.84	24.1
6	7.12	26.8
7	9.69	27.2
8	11.6	29.4
9	8.24	31.2
10	6.31	25.6
11	4.56	20.4

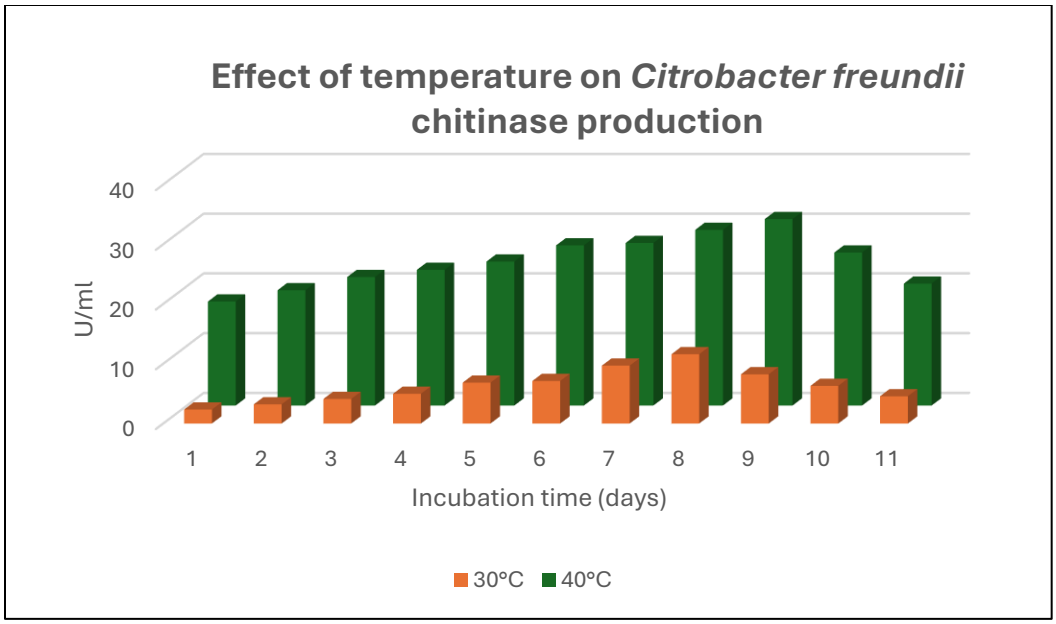


Fig. 2. *Citrobacter freundii* chitinase activity (U/ml) after growth on different temperatures. The microbe was grown on NB medium supplemented with 1% fish wastes.

Effect of pH and salinity on enzyme production

Citrobacter freundii was grown on nutrient broth medium supplemented with 0.5% fish wastes. Split samples of the microbe were allowed to grow for 12 days at 40^{o}C with shaking (180 rpm) on saline nutrient broth and nutrient broth media separately. The extracellular enzyme showed maximum activity in both media when the pH value was slightly alkaline (pH 8). Activity was almost doubled compared to that obtained in acidic pH media (pH 5.5). Enzyme activity reached more than 34 units per ml after growing the microbe saline medium for the indicated period (table 3) and (figure 3).

On the other hand, salt concentration dramatically affects enzymatic activity. The bioremediation process was quietly induced when the microbe was grown on a medium that contained 20g NaCl per liter, activity was at least doubled compared to that obtained when the microbe was grown on a medium that contained 5g NaCl per liter at different pH as shown in (table 3) and (figure 3).

Table 3. Effect of pH and salinity on production of *Citrobacter freundii* extracellular chitinase.

Enzyme activity (U/ml) after growing on:	**pH**			
	5.5	**7.0**	**8.0**	**9.0**
Medium 1	15.9	28.8	34.4	27.5
Medium 2	4.5	12.4	15.4	11.1

- Medium 1 was nutrient broth that contained 15g NaCl per liter
- Medium 2 was nutrient broth that contained 5g NaCl per liter

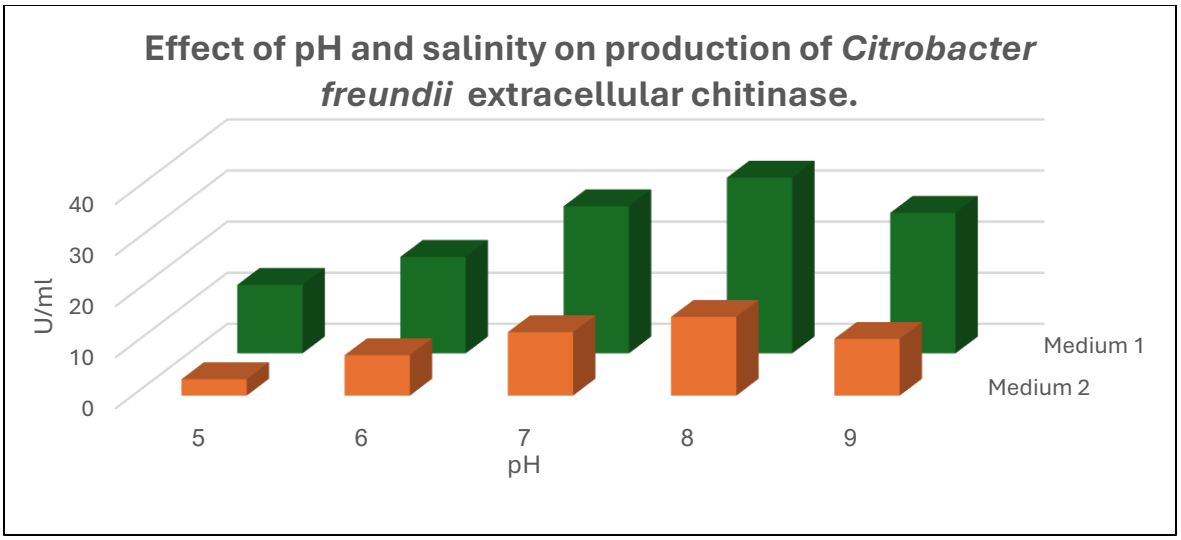


Fig. 3. *Citrobacter freundii* extracellular chitinase activity after growing on (medium 1) nutrient broth medium supplemented with 15 g NaCl per liter and (medium 2) nutrient broth medium supplemented with 5 g NaCl per liter at different pH values.

Effect of different waste concentration on enzyme production

To investigate the effect of waste concentration on the whole bioremediation process of fish waste using *Citrobacter freundii*, the microbe was grown on basal minimal broth mediumII supplemented with different concentrations of dried fesh waste. Maximum extracellular chitinase activity was obtained when 0.5% fish waste was used as shown on table (4).

Table 4. *Citrobacter freundii* chitinase activity (U/ml) after growth on different fish waste concentrations.

	Waste concentration %					
	0.3	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**
Enzyme activity U/ml	1.8	4.2	7.8	3.9	2.5	1.2

Purification of extracellular chitinase

Chitinase produced by *Citrobacter freundii* has many commercial applications including several medical and agricultural uses. It has antibacterial and antifungal properties. It is also considered one of the most active agents that control fungal pathogens. Moreover, chitinase enzymes have a great anticancer effect (Gomaa, 2021).

The extracellular enzyme was purified by salting out using ammonium sulfate at 65% concentration as mentioned by (Nooralabettu, 2014). The precipitated enzyme sample was dialyzed overnight against 0.1 M Tris-HCl buffer followed by application on Sephadex G-100 gel filtration column. Purified enzyme activity reached more than 85 times purification fold after sample application on the gel filtration column (Table 5).

Fractions of Sephadex G-100 gel filtration column were tested for *Citrobacter freundii* extracellular chitinase activity. All active fractions were concentrated in one peak that showed the chitinolytic activity as shown in figure (4).

Table 5. Purification table of *Citrobacter freundii* extracellular chitinase.

Purification Step	Protein test (mg/ml)	Enzyme activity (U/ml)	Specific Activity	Purification fold
Crude solution	12.4	31.6	2.6	1.0
Cell free supernatant	6.9	31.2	4.5	1.7
Pellets after $(NH_4)_2SO_4$ ppt.	1.8	68.3	37.1	14.3
Suspension after dialysis	1.0	70.3	68.9	26.5
After Sephadex G-100 column	0.4	93.1	221.6	85.2

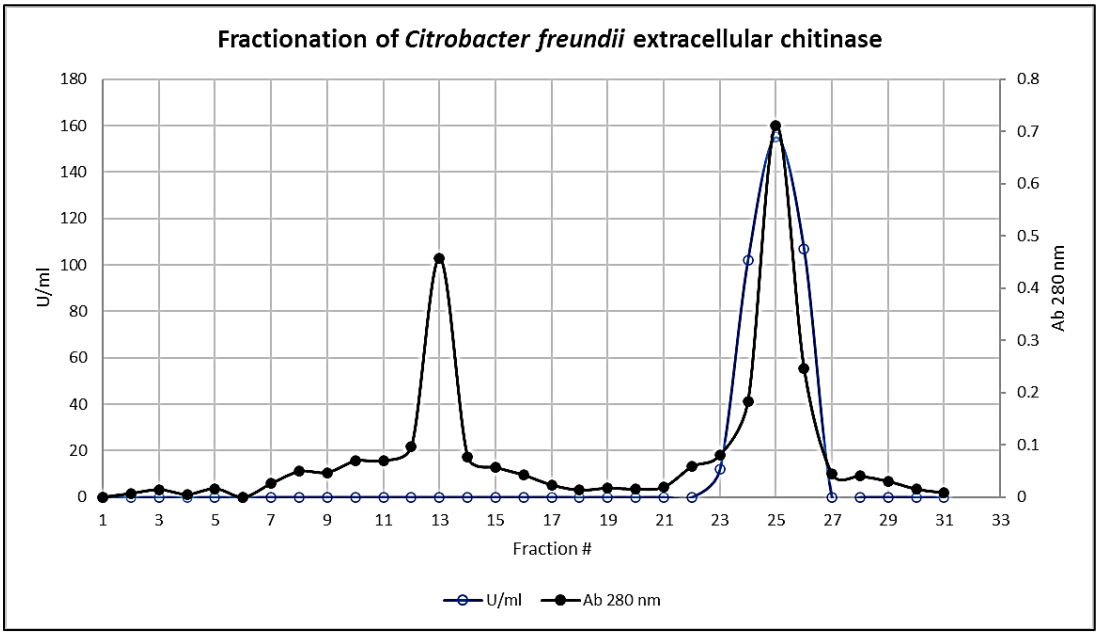


Fig. 4. Fractionation pattern of *Citrobacter freundii* extracellular chitinase. After dialysis, purified enzyme was applied to Sephadex G-100 column.

Enzyme-Nanoparticles preparations

New research trends are now dealing with enzymatic preparations that dramatically enhance the production, chemical and physical characteristics and stability of enzyme preparations. Enzyme nanotechnology is now one of these fields that pay great attention to enzyme stability, especially in environmental and industrial applications. These nanoparticles attain superior solidity in an aqueous solution because of co-precipitation or by adding proteinaceous and bioactive elements onto the outer face of the nanoparticles. (Mahanty et al., 2020**)** The performance of such biogenic nanoparticles gives a boost to cost-effective and sustainable production from industries (Noman et al., 2020). Several research protocols delt with enzyme immobilization through enzyme-nanoparticles formation. These preparations have many advantages affecting the

enzymatic structure, activity and/or stability.

In this study, a split purified *Citrobacter freundii* extracellular chitinase sample was prepared with previously prepared ferric oxide nanoparticles. Enzyme thermal stability of purified enzyme sample was tested simultaneously with enzyme nanoparticle preparation to test the efficiency of the whole nanotechnological process.

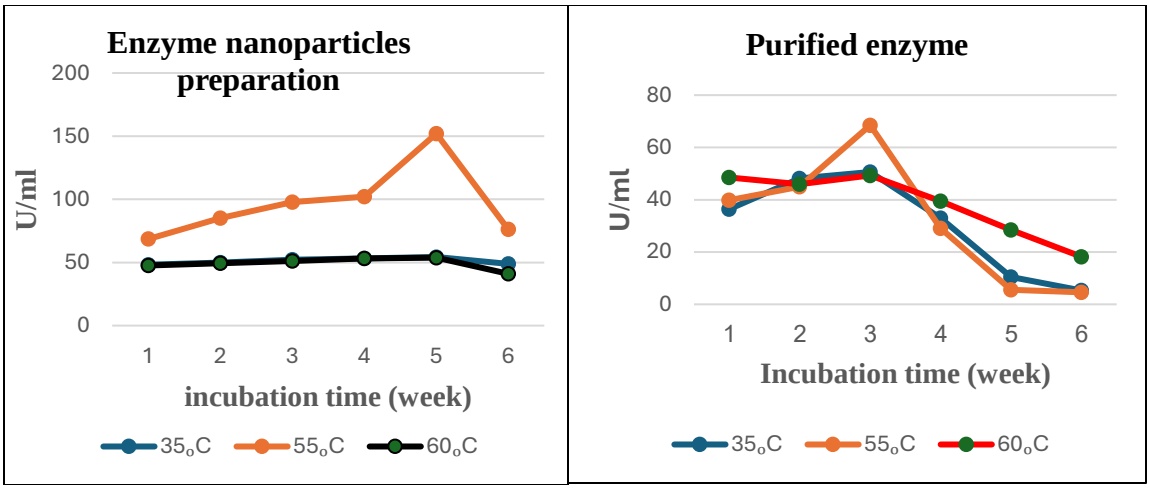
Stability of chitinase nanoparticles

The activity of *Citrobacter freundii* extracellular chitinase was greatly affected after nanoparticle formation. Data in (table 6) and (figure 4) (b) showed that at 35^{o}C, enzyme activity remained almost stable for more than 40 days of incubation. Enzyme stability was reduced to 25% after 6 weeks of incubation. Enzyme immobilization kept more than 90% of this stability for more than 6 weeks at the same temperature (figure 4) (a). At 55^{o}C both purified and immobilized enzyme samples reached their maximum activities. Moreover, enzyme-nanoparticles preparation reached more than 150 U/ml after five weeks of incubation, that's almost more than twice the activity of the purified enzyme at the same temperature (table 6).

Finally, data in table (6) and figures (4) showed that purified *Citrobacter freundii* extracellular chitinase activity was greatly enhanced after enzyme ferric oxide nanoparticle preparation. This character provides very valuable information especially in environmental and industrial applications of the enzyme where the whole solution may be subjected to a variety of different aqueous stresses (like high temperatures and pH values).

Table 6. *Citrobacter freundii* extracellular chitinase activity at different storage time intervals. Purified enzyme sample was immobilized on ferric oxide nanoparticles in a split sample. All activity data for both samples was carried out at the same reaction conditions.

Storage time (week)	***C. freundii* chitinase nanoparticles**			***C. freundii* chitinase**		
	35^{o}C	**55^{o}C**	**60^{o}C**	**35^{o}C**	**55^{o}C**	**60^{o}C**
1	48.2	68.5	47.7	36.4	39.8	48.5
2	49.9	85.1	49.3	48.2	45.0	46.0
3	52.1	97.8	51.1	50.6	68.5	49.3
4	53.2	102	53.2	33.0	29.0	39.4
5	54.3	152	53.8	10.4	5.6	28.5
6	48.8	76.1	41.0	5.3	4.6	18.2



(a) **(b)**

Fig. 4. *Citrobacter freundii* extracellular chitinase activity after incubating the enzyme at different temperature degrees. (a): Purified enzyme sample was immobilized on ferric oxide nanoparticles and (b): Purified *Citrobacter freundii* extracellular chitinase. Activity after incubating both samples was tested at different temperature degrees for the indicated time intervals.

CONCLUSION

In an attempt to produce bacterial extracellular chitinase from the marine bacterium *Citrobacter freundii*. The study focused on bioremediation of fish wastes as good environmental pollutant that could be used as a good source for enzyme production. This indicates the potential of utilizing organic waste as a nutrient source for microbial enzyme production, promoting sustainable practices in waste management. The enzyme demonstrated a remarkable increase in activity at elevated temperatures, particularly at 40 °C. This thermophilic nature suggests that the enzyme can be effectively employed in high-temperature bioremediation processes, enhancing its applicability in industrial settings. The purification process yielded over 85-fold enhancement in enzyme purity, with the enzyme retaining more than 70% of its activity at 60 °C and pH 8.0. Such stability under varying conditions is crucial for its potential industrial applications, where enzymes are often exposed to extreme environments. Enzyme immobilization on ferric oxide nanoparticles significantly improved the enzyme's activity, with the enzyme-nanoparticle preparation exhibiting more than double the activity of the purified enzyme alone. This finding highlights the potential of nanotechnology in enhancing enzyme performance, paving the way for innovative applications in various fields.

The use of *Citrobacter freundii* for chitinase production not only aids in the degradation of chitinous waste but also contributes to reducing organic pollution in aquatic environments. This aligns with the goals of sustainable environmental management and underscores the importance of biotechnological approaches in addressing ecological challenges. Results advocate for further exploration of microbial enzymes and their enhancement through nanotechnology,

contributing to advancements in green biotechnology and sustainable practices.

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