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# Purification and Characterization of Cellulase from Lactobacillus for Enhanced Essential Oil Extraction

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**Abstract:** The objective of the study is to isolate and identify lactic acid bacteria *Lactobacillus* from various local sources and to purify, screen, identify, and assess their efficiency in producing the Cellulase enzyme. The most effective isolates for producing the Cellulase enzyme were identified as fish isolate 3. Purification was accomplished through sequential ammonium sulfate precipitation (40-80%) and dialysis, achieving a specific activity of 59.51 $U.mg^{-1}$. This was followed by gel filtration chromatography using a Superdex 200 column on an ÄKTA Pure 25 apparatus, resulting in a purification fold of 20.39 and a yield of 11.49%. The molecular weight of Cellulase was determined to be 36 kDa using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The optimum pH for both the purified and partially purified enzyme activity and stability was found to be 5. The optimum temperature for enzyme activity was 55°C for both the completely purified and partially purified enzymes. The temperature range for enzyme stability was between 25-45°C for the partially purified enzyme and between 25-55°C for the completely purified enzyme. Furthermore, the partially purified enzyme was utilized to enhance the efficiency of extracting essential oils from Star Anise (*Illicium verum*).

**Keywords**: Cellulase, Characterization, Essential oils, Lactobacillus, Purification.

## Introduction

Enzymes are biological molecules, also known as "biocatalysts," produced by all living organisms. The demand for enzymes has grown significantly due to recent industrial advancements favoring green chemistry to replace harmful chemicals. For large-scale chemical production, enzyme synthesis requires affordable and readily available substrates. Plant cell walls are a key carbon source, containing sugars that microbes can use for energy and carbon. Microbial enzymes break down these sugars into digestible components. The resulting sugars and materials serve as nutrients for plants, animals, and the organisms themselves (Maghraby *et al.*, 2023 & Rahman *et al.* ,2014).

Lactic acid bacteria (LAB) are important microorganisms that produce lactic acid primarily as a by-product during metabolic activities and play multifaceted roles in the agricultural, food and clinical sectors. Lactic acid bacteria are used in many fermentation processes, as the use of these bacteria is considered one of the most traditional and used sciences in food processing and preservation. Due to the importance of lactic acid bacteria in many food applications and because they have therapeutic properties to enhance human health, research is ongoing to obtain strains with properties that enhance the quality of food products (Bintsis,2018; Al-Seraih *et al.,* 2022) .

Microbial enzyme manufacturing has recently gained popularity. Microbial enzymes, particularly cellulase, are commercially exploited in a variety of fields. These include the agriculture, fermentation, paper, and textile sectors (Msakni *et al.,* 2024). Bacterial cellulases have shown great potential for use in various industries. These include textiles, paper, fermentation, agriculture, and biofuels. In the food industry, cellulase has multiple applications. It is used to soften fruits, filter fruit juices, and reduce dough roughness. It also helps extract oils, polyphenols, and carotenoids from plant sources (Ejaz *et al.,* 2021). Cellulase is the enzyme that degrades cellulose. Cellulases are a collection of three enzymes (EC 3.2.1.4 Endo-glucanases, EC 3.2.1.91 Exo-glucanases, and EC 3.2.1.21 glucosidase) that hydrolyse β 1-4 glycosidic linkages (Shyaula *et al.,* 2023). Cellulase is a widely used, commercially available industrial enzyme. It is produced by various microorganisms, including bacteria and fungi. These microbes produce cellulase when grown on cellulosic materials. Cellulase converts cellulose into simple sugars. These sugars are useful in many industrial fermentations. These fermentations produce valuable materials (Doan *et al.,* 2024). Lactobacilli bacteria, particularly L. plantarum, are known to produce diverse metabolic products. These include lactic acid, hydrogen peroxide, bacteriocins, bioactive compounds, and enzymes (Khalil *et al.,* 2021; Al-Salhi *et al.,*2022).

This study aimed to produce cellulase enzymes from a local bacterial isolate. The goal was to enhance the extraction efficiency of essential oils from plants.

## Materials & Methods

### Isolation, production and extraction of the enzyme

Fifteen samples were collected from various sources, including fruits, soil, yoghurt, pickles, and fish, to isolate lactic acid bacteria. Microorganisms were isolated from live fish viscera, with fish sample 3 being the best for cellulase enzyme production. Bacteria were grown on a nutrient medium composed of FeSO4.7H2O (0.0004%), CACl2.2H2O (0.0001%), MgSO4.7H2O (0.02%), tryptone (0.20%), KH2PO4 (0.4%), Na2HPO4 (0.04%), and CMC (1.0%). The pH was adjusted to 7, and the medium was sterilized at 121°C for 15 minutes. Each 250 ml flask contained 50 ml of medium, inoculated with 2.5 ml (5%) of an 18-24-hour bacterial isolate. Incubation occurred in a shaking incubator at 37°C for 48 hours at 150 rpm. Post-incubation, the medium was centrifuged at 6000 rpm for 10 minutes to remove microorganisms and residues, leaving the filtrate as the crude enzyme source (Shaikh *et al.*, 2013).

### Enzyme Purification

The enzyme was concentrated using ammonium sulphate to a saturation rate of 40-80%. After that, a cooled centrifugation process was run for a quarter of an hour at 1000 rpm and then Dialysis bags with molecular weights ranging between (12-14) kilodaltons were used after activation to carry out the membrane osmosis process of the enzyme for 24 hours against distilled water, with the water being changed every 6 hours (Hamdan & Jasim 2018; Al-Temim *et al* .,2023) .

### Gel Filtration

The ÄKTA Pure 25 apparatus with a Superdex 200 10/300 GL column (10 mm diameter, column size 23.562 ml, pressure 1.5 mpa, dimensions 10 × 300 mm, flow rate of 0.5 ml.min$^{-1}$) packed with agarose and dextran. The column was calibrated using two litre of acetate buffer pH 6. The concentrated sample (0.5 ml) was then injected gradually after being filtered by a millipore filter with a 0.45 µm filter and monitored for distinct peaks at 280 nm. The enzymatic activity (U.ml$^{-1}$) was then measured (Elsababty et al., 2022)

### Determination of Enzyme Purity

The enzyme's purity was assessed using polyacrylamide gel electrophoresis under non-denaturing conditions. The method followed Laemmli (1970) and Garfin (1990). A slab electrophoresis device was used with polyacrylamide gel, without the denaturing agent SDS.

### Characterization of the Pure Enzyme

✓ The molecular weight was determined using the Laemmli method of electrophoresis in a polyacrylamide gel with sodium dodecyl sulphate (SDS) present, as described by Laemmli (1970) and further detailed by Garfin (1990). Using the method of gel filtration and in another way based on the relative mobility (Rm) of the protein bonds Standard and for the enzyme and extract the molecular weight of the enzyme by plotting the relationship between the logarithm of the molecular weights of standard proteins against their relative movement in the gel.

$$Rm = \quad Migration\ distance\ of\ the\ band\ /\ Migration\ distance\ of\ the\ front$$

✓ Ideal pH for Stability and Enzyme Activity: The ideal pH for stability was investigated by measuring the remaining activity (%) using various buffer solutions within the pH range of 3-8 at 35°C for 30 minutes, utilizing cellulose as a substrate. The optimal pH for enzymatic activity (U.ml$^{-1}$) was determined using sodium acetate buffer within the pH range of 3-5 and phosphate buffer within the pH range of 6-8, both at a concentration of 0.2 M.

✓ Ideal Temperature for Stability and Enzyme Activity: The ideal temperature for stability was assessed by incubating cellulose in a water bath at temperatures ranging from 20°C to 80°C for 30 minutes, followed by immediate cooling in an ice bath. To determine the optimal temperature for enzymatic activity, the enzyme was incubated in a water bath at temperatures ranging from 25°C to 75°C for 30 minutes.

### Enzyme application in extracting essential oil from star anise

The dried fruits of star anise (*Illicium verum*). were purchased from Busrah City's local market. The samples were cleaned, crushed and ground with an electric grinder and stored in glass bottles until use.

### Method

The essential oil was extracted from star anise using steam distillation with a Clevenger apparatus (Yousif &Hassan,2023). Two samples were prepared, each weighing 50 grams of star anise.

- ✓ The first sample was mixed with 500 ml of distilled water Aloush &Salman (2016).
- ✓ The second sample was mixed with 200 ml of sodium phosphate buffer solution (pH 6, 0.2 M) containing 5 ml of partially purified cellulase enzyme.

The second sample was placed on a magnetic stirrer for 6 hours. After stirring, distilled water was added to bring the volume to 500 ml. Both samples were then placed in the Clevenger apparatus. The extraction process lasted 4 hours, ensuring complete oil extraction. The extracted oil was collected in small, opaque, tightly sealed glass bottles. Both samples were stored in a refrigerator at 4°C. (Wanger *et al*., 2009; Yu *et al*., 2019).

### Calculating the Amount of Extracted Essential Oil

The extracted star anise essential oil yield for the two samples was determined by dividing the volume of extracted oil by the sample weight prior to extraction. The resulting ratio was computed as follows: The extracted oil ratio is calculated by dividing the volume of oil by the sample weight by 100.

weight of oil/weight of sample × 100 is the ratio of extracted oil Aloush &Salman (2016).

## Results & Discussion

### Isolation

The results of the study indicate that 15 isolates of lactic lactic acid bacteria Lactobacillus (another research which is part of the requirements for obtaining a master's degree). The isolates obtained varied based on their sources, which included milk, cucumber, banana, watermelon, soil, grapes, fish (four isolates), pickles (three isolates), watercress, and tomatoes. The top-performing isolates were selected based on enzyme effectiveness measurements.

These isolates underwent secondary screening by estimating enzyme effectiveness and specific effectiveness in a liquid medium containing CMC as a carbon source. The medium was prepared, sterilized, and inoculated with 24–48-hour-old active isolates. Three replicates were tested for each isolate.

Fish isolate 3 showed the highest enzymatic effectiveness, with a spectrophotometer absorption of 4.546. It was followed by fish isolate 4 (2.693) and pickle isolate 2 (2.639). The enzymatic effectiveness of other isolates, such as fish 1, pickle 1, and pickle 3, was lower, at 2.169, 1.775, and 2.049, respectively. Fish isolate 3 was chosen as the best for enzyme production (Desa,2008).

**Table (1): lactic acid bacteria isolates producing the enzyme Cellulases**

| Isolate | Enzymatic activity U.ml$^{-1}$ |
|---|---|
| Fish 1 | 2.169 |
| Fish 3 | 4.546 |
| Fish 4 | 2.693 |
| Pickle 1 | 1.775 |
| Pickle 2 | 2.639 |
| Pickle 3 | 2.049 |

### Purification

Ammonium sulfate salts were used to concentrate the cellulase enzyme produced by Lactobacillus bacteria. Saturation rates ranged from (40- 80) %. This process achieved a 5.76-fold purification, a 31.42% enzyme yield, and a specific activity of 59.51 U.mg$^{-1}$.

These results are consistent with previous studies highlighting ammonium sulfate as an effective first step in enzyme purification. Ugras *et al.*, (2024) reported a 3-fold purification and 1.95% yield when concentrating cellulase from Bacillus cereus DU-1 at 80% saturation. Elsababty *et al.* (2022) achieved a 2.04-fold purification and 12.35% yield when concentrating cellulase from Bacillus licheniformis at 20–80% saturation.

**Gel Filtration**

After precipitating the medium extract with ammonium sulfate, protein activity was measured at 280 nm using the Pure 25 AKTA device. Figure (1) shows two distinct peaks. Each peak was collected separately, and its enzymatic activity was measured.

The first peak contained the cellulase enzyme, with an enzymatic activity of 6.53 units.ml⁻¹. The second peak showed no enzymatic activity, indicating the absence of the enzyme. Table 1 shows that the purification fold and enzyme yield were 20.39 and 11.49%, respectively. These results align with previous studies using chromatography for cellulase purification. Vijayaraghavan & Prakash (2012) reported a 14.5-fold purification for cellulase from ***Bacillus sp.*** using gel filtration. Lee *et al.*, (2008) found a 20.8fold purification and 9.4% yield for cellulase from ***Bacillus amyloliquefaciens*** DL-3 using ion exchange chromatography. As shown by Ugras *at el.*, (2024), the possibility of purifying the cellulase enzyme isolated from Bacillus cereus DU-1 by precipitation using ammonium sulfate at a saturation rate of 80%. and the specific activity was 1.95U. mg$^{-1}$ and the number of purification times was 3 times.

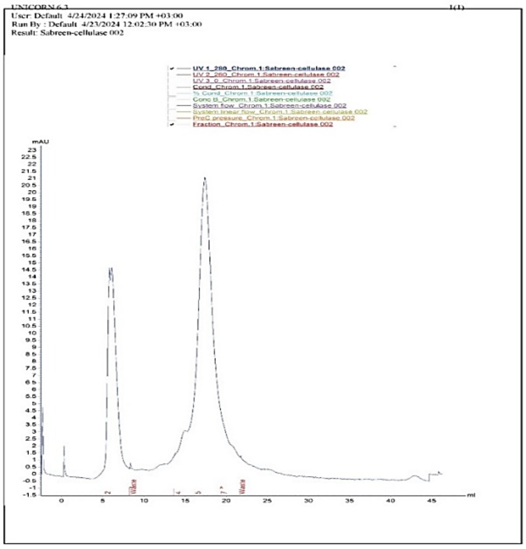

**Fig. (1): Superdex 200 10/300 GL Gel** Filtration Chromatogram of Cellulase Enzyme by ÄKTA Pure 25.

**Table (1): Purification of cellulase from *Lactobacillus***

| Step | Volume ml | Activity U.mL$^{-1}$ | Protein mg.ml$^{-1}$ | Specific Activity U.mg$^{-1}$ | Total Activity Unit | Recovery % | Purification factor |
|---|---|---|---|---|---|---|---|
| Crude extract | 200 | 4.546 | 0.44 | 10.331 | 909.2 | 100 | 1 |
| Saturated ammonium sulphate (40-80)% | 25 | 11.426 | 0.192 | 59.51 | 285.65 | 31.42 | 5.76 |
| Gel filtration sephadex G-200 | 16 | 6.530 | 0.031 | 210.65 | 104.48 | 11.49 | 20.39 |

**Assessing the Purity of Enzymes**

Figure 3 shows the results of ammonium sulfate electrophoresis and Superdex 200 gel filtration. These procedures purified the cellulase enzyme isolated from ***Lactobacillus*** bacteria. Electrophoresis was performed under non-denaturing conditions. This confirmed enzyme purity and the absence of other proteins or enzymes. A single protein band on the gel indicates enzyme purity. The purity determination test showed only one protein band for the enzyme. This indicates that the extraction and purification procedures were effective.

Figure (2) displays acrylamide gel electrophoresis results:

(A) Crude enzyme extract

(B) Enzyme purified by gel filtration (without SDS denaturant).

## Enzyme Characterization

### Determination of Molecular Weight

The molecular weight of the cellulase enzyme was estimated by electrophoresis in polyacrylamide gel in the presence of the denaturant SDS. The relationship between the relative mobility of standard proteins with known molecular weight (Rm), which is used to estimate the molecular weight of the pure enzyme, and the logarithm of the molecular weight is depicted in Figure 4. It was feasible to calculate the enzyme's molecular weight by measuring its relative mobility (Rm), which came out to be equivalent to 36 kilodaltons. This score is higher than what he got Yin *et al*., (2010), when estimating the molecular weight of Cellulase enzyme purified from Bacteria Bacillus subtilis YJ1 , which was 32.5 KDa ,as it was close to what was obtained Ugras et al. (2024), when estimating the molecular weight of Cellulase enzyme purified from Bacteria Bacillus cereus DU-1 by electrophoresis method .The molecular weight 40 KDa. And it was lower then what was obtained Msakni *et al*., (2024), when estimating the molecular weight of Cellulase enzyme purified from Bacteria Bacillus subtilis 171ES , which was 63 KDa .

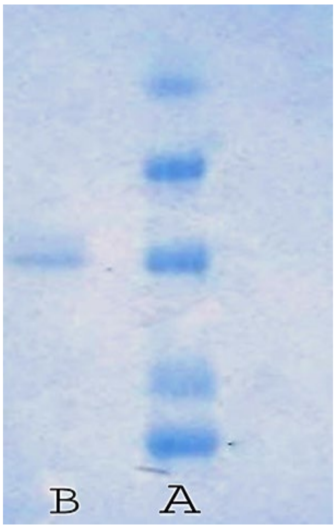

**Fig. (2): Cellulase purity was assessed using electrophoresis in polyacrylamide gel without SDS. A represents crudely extracted enzyme, and B represents pure enzyme following gel filtration.**

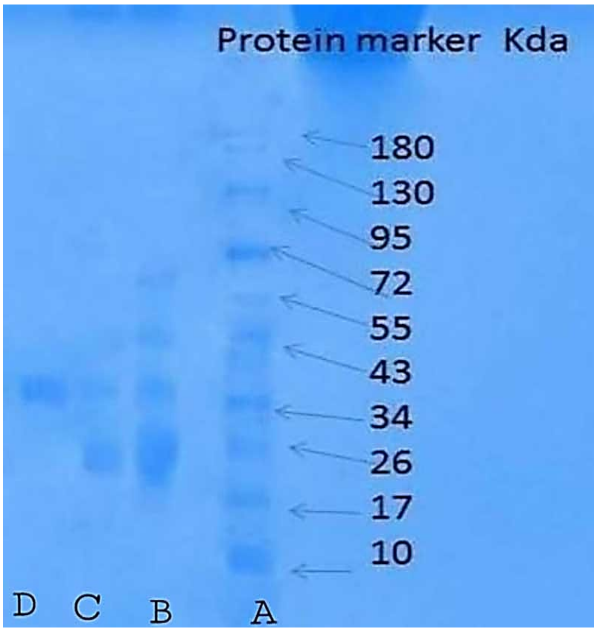

**Fig. (3): Electrophoresis with SDS in polyacrylamide gel, A: standard proteins; B: crude extracted;C: partially purified cellulase;D: pure enzyme**

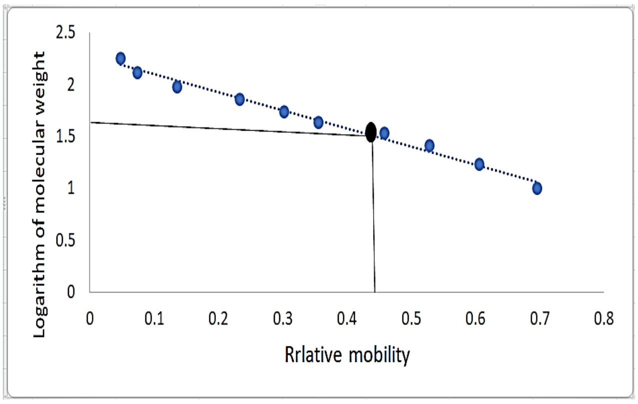

**Fig. (4): Standard curve to determine the molecular weight of cellulase produced from Lactobacillus by SDS-PAGE.**

### Optimal pH for enzyme activity

The optimal pH for the activity of both pure and partially purified cellulase enzymes was determined within a pH range of 3 to 9. Statistical analysis at $p \leq 0.05$ showed significant differences in enzyme activity across pH values.

Figures (5) and (6) reveal that the optimal pH for enzyme activity was 5. At this pH, the pure enzyme showed an activity of 6.53 $U.ml^{-1}$, while the partially purified enzyme reached 11.426 $U.ml^{-1}$.

Enzyme activity decreased as the pH moved away from 5, with the lowest activity observed at pH 3 and 9. This effect may result from the pH of the reaction medium influencing ionizable groups in the enzyme's active site. Alternatively, it could be due to changes in the ionic state of the substrate, the enzyme-substrate complex (ES), or the resulting enzyme-product complex (EP) (Whitaker, 1972; Fullbrook, 1983).

These results align with previous studies. Shyaula *et al*., (2023) reported an optimal pH of 5 for the partially purified cellulase enzyme from Bacillus licheniformis PANG L. Similarly, Odufuwa et al. (2024) found that the optimal pH for the cellulase enzyme purified from Bacillus subtilis was also 5. Additionally,

These results are similar to Pachauri *et al*., (2020), who found the optimum pH for cellulase from Trichoderma longibrachiatum to be 4.8. They also align with Mawadza *et al*., (2000), who reported an optimum pH of 5–6.5 for cellulase from Bacillus sp. HR68.

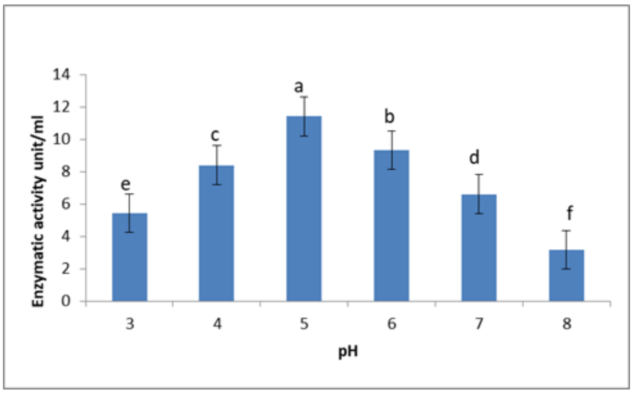

**Fig. (5): Ideal pH for partially refined cellulase**

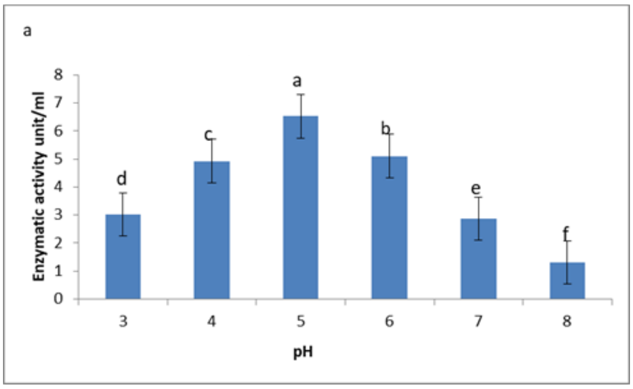

**Fig. (6): Optimal pH for pure cellulase.**

### Optimal pH for enzyme stability

The findings in Figures 7 and 8 indicate that pH 5 is optimal for the stability of fully and partially purified cellulase enzymes. Statistical analysis shows that pH values varied significantly at the p≤0.05 level. The enzyme's stability decreased at other pH values but remained 75.2% and 17.88% active at pH 6. This decline occurs because pH affects the enzyme’s secondary and tertiary structures, altering the active site and causing denaturation, which reduces activity (Segel, 1976). The results align with Doan *et al.*, (2024). They reported an optimal pH of 5-7 for cellulase stability in *Paenibacillus elgii*.

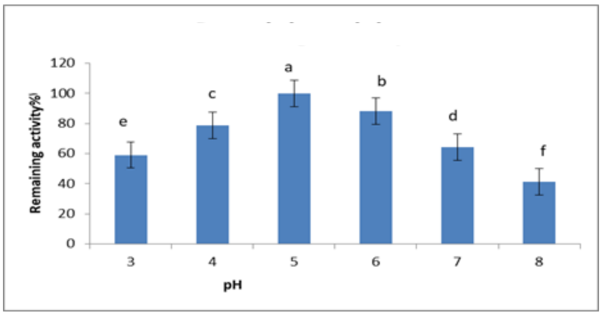

**Fig. (7): Optimum pH for partial stability of cellulose**

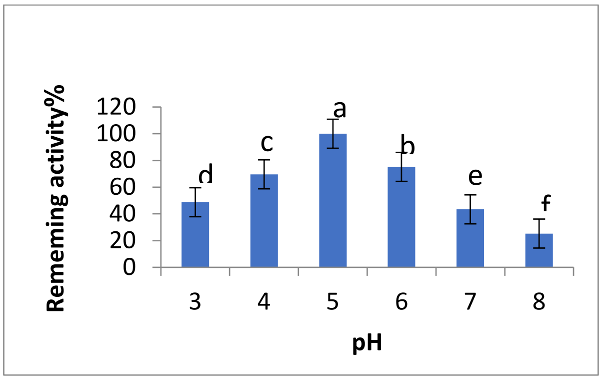

**Fig. (8): pH of pure cellulase stability**

### Temperature on enzyme activity

The impact of temperature on the activity of pure and partially purified cellulase is depicted in Figures 9 and 10, respectively. At 55°C, the enzyme activity reaches its highest, reaching 9.23 and 13.175 unit.ml$^{-1}$, respectively. It is observed that the enzyme activity increases with temperature. The statistical analysis showed that the enzyme's temperature varied significantly at a p-value below 0.05. It then began to decline gradually at 75 and 85 m.

Enzyme reactions accelerate with temperature increases up to a certain point. This is due to more collisions between enzyme molecules and the substrate, caused by increased kinetic energy from the temperature rise.

Enzyme activity drops at high temperatures because reacting molecules absorb excessive energy. This alters the enzyme's tertiary structure, causing deformation and partial loss of function (Segel, 1976).

These results align closely with findings by Odufuwa *et al.*, (2024) and Msakni *et al.*, (2024). They estimated the optimal temperature for the cellulase enzyme from *Bacillus subtilis* bacteria to be 50°C. Similarly, Shyaula *et al.*, (2023) found the optimal temperature for the cellulase enzyme from *Bacillus licheniformis* PANG L bacteria to be 60°C. These results were similar to those of Ugras *et al.*, (2024). They estimated the optimal temperature for the cellulase enzyme from *Bacillus cereus* DU-1 bacteria to be 50°C.

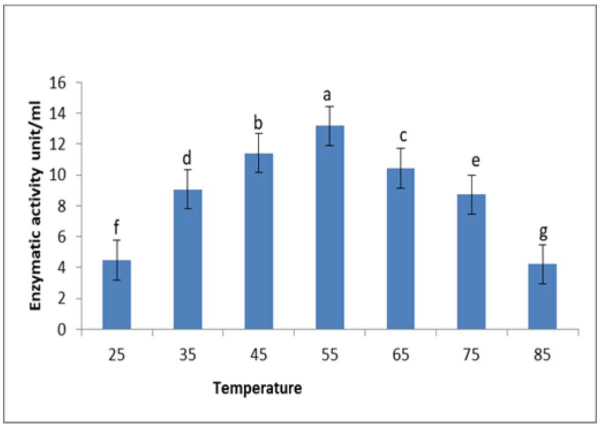

**Fig. (9): Optimum temperature for partial cellulase**

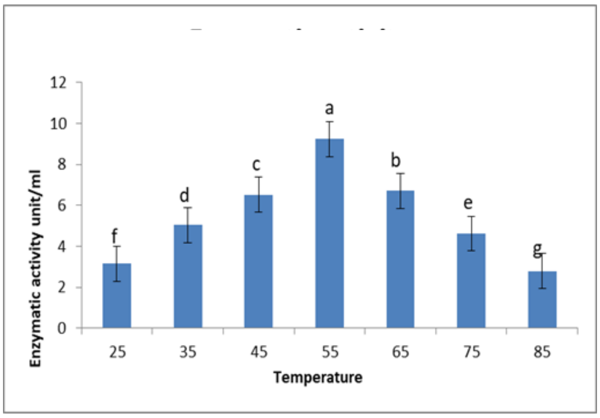

**Fig. (10): Optimum temperature for cellulase for total purification.**

### Thermal stability of the enzyme

Figure 11 shows the effect of temperature (20-80°C) on cellulase enzyme activity. Pure and partially purified enzymes were incubated for 30 minutes. Significant differences in activity were observed between temperatures ($p \leq 0.05$). The pure enzyme was fully effective between 25-45°C. The partially purified enzyme was fully effective between 25-55°C. Effectiveness decreased at higher temperatures. At 85°C, the pure enzyme's activity was 26.63 U .ml$^{-1}$. At 85°C, the partially purified enzyme's activity was 46.55 U .ml$^{-1}$. The partially purified enzyme was more stable. Other materials present likely protected it. This protection allowed it to resist higher temperatures (Segel, 1976). These results are similar to Yin *et al.* (2010). They found a 20-50°C stability range for cellulase from Bacillus subtilis YJ1. Our results are higher than Shyaula *et al.*, (2023). They reported a 30-45°C optimum for cellulase stability in Bacillus licheniformis PANG L. Our results also agree with Mawadza *et al.*, (2000). They found a 30-50°C stability range for cellulase from Bacillus sp. HR68.

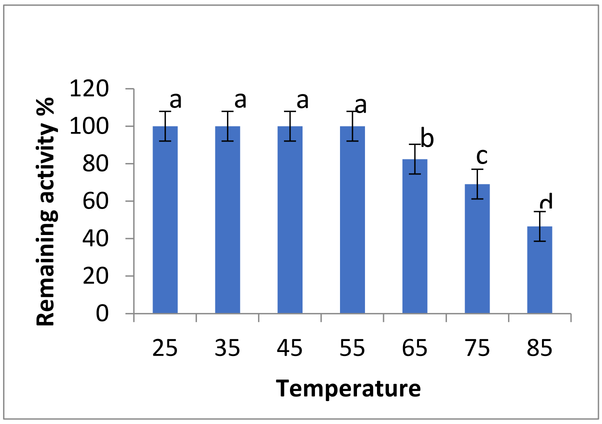

**Fig. (11): Optimum temperature for partial**

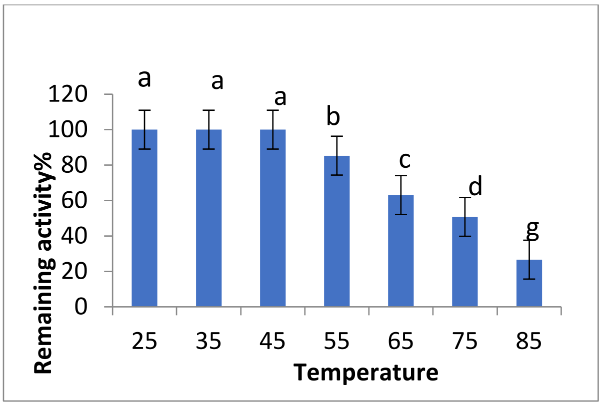

**Fig. (12) Optimum temperature for a stability of pure enzyme of pure cellulase**

### Extraction of essential oils

The yield essential oil extraction from star anise, with and without cellulase it was7%, 3% respectively. Adding the enzyme increased essential oil extraction efficiency, these results align with Wainer *et al.*, (2022), who used cellulase enzyme to extract essential oil from Lavandula x intermedia. They observed higher extraction efficiency compared to other methods. The results also match Iko *et al.,* (2022), who found increased extraction yield from nutmeg seeds treated with cellulase enzyme compared to untreated samples

## Conclusion

In this study, Lactobacillus buchneri bacteria were isolated from the intestines of live fish. It was observed that these bacteria are capable of secreting cellulase, an enzyme of significant industrial importance following proteases. The purification process involved two steps: first, precipitation with ammonium sulfate to obtain a partially purified enzyme, and second, gel filtration using the AKTA pure system to achieve a pure enzyme. Purification and characterization of the enzyme are essential to evaluate its economic viability and potential industrial applications, such as improving the quality of baked goods and enhancing the efficiency of essential oil extraction.

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## Contributions of authors

Authors 1, 2, and 3 Constructed the idea Collection of specimens, Laboratory techniques, wrote and revised the manuscript.

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## Conflict of Interest

All authors declares no conflict of interests

## Ethics approval

Not applicable for this section

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# تنقية وتوصيف انزيم Cellulase من بكتريا *Lactobacillus* واستخدامه في تحسين كفاءة استخلاص الزيوت العطرية

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**المستخلص:** تهدف هذه الدراسة إلى عزل وتشخيص بكتيريا حامض اللاكتيك من مصادر محلية مختلفة ثم تنقيتها وتشخيصها وتحديد كفاءتها في إنتاج إنزيمCellulase ، وجد ان أفضل عزلة لإنتاج إنزيم Cellulaseكانت عزلة السمك 3. ركز بعد ذلك المستخلص الانزيمي الخام بأملاح كبريتات الأمونيوم بنسبة (40–80%)، ثم اجريت عملية الديلزة وقدرت الفعالية النوعية للأنزيم المنقى جزئيا وكانت 59.51 (وحدة. مل$^{-1}$)، ثم مرر بعمود الترشيح الهلامي Superdex 200 بجهاز ÄKTA Pure 25 للحصول على الانزيم النقي وقد بلغت عدد مرات التنقية والحصيلة 20.39، 11.49%على التوالي. قدر الوزن الجزيئي للإنزيم بطريقة الترحيل الكهربائي بوجود المادة الماسخة (SDS)، اذ بلغ 36 كيلو دالتون، ووجد ان الرقم الهيدروجيني الامثل لفعالية وثبات الإنزيم النقي والمنقى جزئيا كان 5، بينما كانت درجة الحرارة المثلى لفعالية الإنزيم النقي والمنقى جزئيا 55 م ودرجة الحرارة المثلى لثبات الانزيم النقي والمنقى جزئيا بلغت 25–45 م ،25–55 م على التوالي. تم تطبيق الإنزيم المنقى جزئيًا في تحسين كفاءة استخلاص الزيوت العطرية من نبات اليانسون النجمي (Illicum verum).

الكلمات المفتاحية: Cellulase، التوصيف، الزيوت العطرية،*Lactobacillus* ، التنقية.