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Extraction, Partial Purification and Characterization of Lipase Enzyme from Different Animal and Plant Sources

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Abstract

The study aimed to extract the lipase enzyme from different sources, estimate its enzymatic activity, choose the best sources in terms of enzymatic activity, partially purify, and characterize it. The enzyme was extracted from plant sources (pumpkin seeds, melon seeds, and orange peels). The enzyme extract was precipitated with ammonium sulfate at a saturation level of 80%. The dialysis and lyophilization processes were performed for the partially purified enzyme. The optimum temperature for the partially purified enzyme was 40°C and the optimum pH was 7. The enzyme activity of the plant sources was 2.598, 0.723, and 1.333 units g^{-1}, respectively. Animal sources (chicken pancreas and intestinal tract of carp fish). The enzymatic activity of the animal sources was 4.122 and 6.532 units g^{-1}, respectively. The highest activity was in the intestinal tract of carp fish. The protein concentration was 4.212 mg mL^{-1}, the total activity was 1658.88 units, the number of purification times was 1.638 times, and the enzyme yield was 80.18% using tributyrin as a substrate. It can also be noted that the enzyme produced from the intestinal tract of common carp, which was rich in it, can be used in various industrial fields.

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Introduction

Enzymes are biological catalysts, which are complex catalytic proteins with exceptional catalytic power. They also have high specificity and are essential for all forms of life by catalyzing chemical reactions in cells. They are divided into seven main groups (Tao *et al.*, 2020). They are used in many industries, including the food industry (Ahmad *et al*., 2018). As well, they are used in dairy products, bread, and beverages including juices, wine, and beer (Taheri-Kafrani *et al*., 2021). Lipases (EC 3.1.1.3) are enzymes that belong to the hydrolytic enzymes. The triacylglycerol acyl hydrolases (E.C. 3.1.1.3) are the cluster of enzymes used to hydrolyze the ester bond of triglycerides, diglycerides, and monoglycerides into fatty acids and glycerol (Odeh and Khalifa, 2019). They contain the serine hydrolase class and do not require a cofactor to function. It has different substrates and produces glycerol and free fatty acids through the hydrolysis of fats and oils. It has broken between ester bonds and produced mono- and di-fats, free fatty acids, and glycerol. Due to its wide applications and the simplicity with which it can be produced in large quantities, it is an important group of enzymes in terms of biotechnology (Sood *et al*., 2023).

Enzymatic modification is considered the safest method to obtain excellent and accurate functional changes in industrial applications (Jasimand Nasser, 2023). It has been found that the activity of the enzyme is affected by different factors, such as temperature, enzyme concentration, pH, metals, reaction time, activators, inhibitors,

and substrate concentration (Al-Haidari *et al*., 2021). Enzymatic catalysis in lipase also represents a remarkable phenomenon that facilitates reaction processes under ambient manufacturing conditions, with the possibility of enhancing specificity and speed due to the enzyme's stability over a wide range of temperatures and pH. This approach has played a major role in many industrial fields, contributing significantly to the intensification of production processes (Hussain *et al*., 2023).

Lipase enzymes are available in nature (Jassim and Al-Amery, 2019), and they have the ability to hydrolyze fats into fatty acids and glycerol at the water lipid interface. It can also reverse the reaction in non-aqueous media. This distinctive natural ability makes it the most widely used in various industrial applications (Patel*et al*., 2019). It has also been used to produce lean meat by removing fat from fish and meat products. In addition, lipases are used in bread, as they can improve the rheology of the dough and increase its size, strength, and stability (Auda and Khalifa, 2019). The lipase enzyme is also of particular importance because it is able to work on low-quality raw materials under moderate conditions (Baena *et al*., 2022). These enzymes also have many applications in many industries such as leather, food, soap, detergents, medicines, textiles and biofuels. They are present in many living organisms such as animals, and plants, fungi, and bacteria (Ali *et al*., 2021b). The lipase enzyme can be obtained from animals, plants, as well as many bacteria (Jabour and Odeh, 2020) and yeasts and fungi (Chechan *et al*., 2020).

They are stable enough that these enzymes are considered natural catalysts, but commercially, only lipases from microorganisms are largely used (Mossawi *et al*., 2016). This is due to genetic manipulation and direction of microorganisms into production (Adetunjiand Olaniran, 2021). Despite the wide range of microbial lipases (Ali and Muslim, 2020), the use of these enzymes on a large industrial scale is still limited due to high production costs, which led to the search for other diverse sources of the enzyme. Industrial demand for new sources with different catalyst properties has motivated researchers to choose new sources (Ali *et al*., 2021a). One of the places that could be an alternative to enzyme production is plants (Hui, and Leong, 2018), as many plant tissues and parts, including seeds, leaves, fruits, and peels showed the presence of lipase, including the highest concentration of lipase in seeds. However, other plant sources have also shown a decent amount of lipase activity. The lipase found in oilseeds has great potential for commercial and industrial exploitation. The demand for lipolytic enzymes has increased due to the possibility of using it in various manufacturing processes of industrial goods such as foodstuffs and pharmaceuticals (Okunwaye *et al.*, 2015). They are also effective and have promising applications in nutrition, food and industry. This is because they can provide high-income, pure products under achievable reaction conditions. They are an environmentally friendly option. The importance lies in producing high-value compounds such as fatty acid esters. As well as the potential to be used as flavoring agents, antioxidants or antimicrobial agents, in addition to structured lipids that offer specific functional properties not found in nature (Reyes-Reyes *et al*., 2022).

The pancreas of local chickens and the common carp *Cyprinus carpio* represent the animal sources. It is considered one of the most common types of fish available locally, and such types of fish are cultured in Iraq because their many characteristics make them suitable for reproduction in the aquatic environment in Iraq. They are expected sources of the enzyme (Karim *et al*., 2022). Lipases also play a major role in industrial processes as catalytic enzymes for biological applications, which are safer and more environmentally friendly. It has been used in food as a result of its specialized and useful functions in processing and improving the quality of products.

Studies conducted on the lipase enzyme indicate that the enzyme is very active in decomposing long-chain fatty acids. As well as, its work in a wide range of temperatures and pH (Ali *et al*., 2022a). It must also be applied before conducting any enzymatic study to know the biochemical properties of the enzyme. Therefore, this study aimed to extract the lipase enzyme from different sources, choose the best one, partially purify it, and characterize it.

Materials and Methods

Sample collection

The samples of *Cyprinus carpio* were collected from local markets in Baghdad City for the period 10-18 September 2023. For samples of the pancreas of local chickens *Gallus gallus* localus, were collected from local markets in Kut City for the period of 1-8 September 2023. *Citrus Sinensis* orange peels were collected from the local markets in Kut City for the period September 22-26, 2023, and pumpkin *Cucurbita Pepo* from Local markets in Kut City for the period 18-22 September 2023. Samples of the seeds of the local melon *Citrullus Lanatus* were also collected from Samarra City for the period of 24-28 September 2023.

Extraction of lipase enzyme from the intestinal tract of carp

The enzyme was extracted from carp according to the method (Naser, 2010). The intestinal tract of the carp was cleaned with cold distilled water to remove fats and unwanted substances. A 0.2 M potassium phosphate solution, pH 7, was added at a mixing ratio of 1 from the intestinal tract of the carp to 4 from a 0.2 M potassium phosphate solution, mixed with an electric mixer for 2 mins, and filtered with Whiteman filter paper No. 1, after which the extract was centrifuged at 5000 x g for 30 mins at 4 °C. The supernatant was classified as a crude enzyme.

Extraction of the lipase from chicken pancreas

Extraction of the enzyme from chicken pancreas was according to (Sandhya and Nagamani, 2020). Twenty grams of chicken pancreas were homogenized with 20 ml of sodium phosphate 0.1 M pH 7 in a mortar and pestle until homogeneous. The filtration process was applied using Whiteman No. 1 filter paper with a Buchner funnel, then the refrigerated centrifugation process was used at a temperature of 4 °C at 6000 x g for 10 minutes, and then the filtrate was taken as a crude enzyme.

Extraction of the lipase enzyme from seeds

Extracting the enzyme from the seeds of pumpkin and melon was according to (Al-Haidari *et al.,* 2020). A quantity of pumpkin and melon seeds was taken, and the germination process was applied for 72 hours. The first step is to take the seeds, remove the developing buds, and mix them with 0.1 M sodium phosphate buffer solution, pH 7, with a mixing ratio of 1 pumpkin seed extract to 6 M sodium phosphate solution. Then, it was filtered with Whiteman No. 1 filter paper, and the refrigerated centrifugation was carried out at 10,000 xg for 30 min at a temperature of 4 °C, and the filtrate was taken as a crude enzyme**.**

Extraction of lipase enzyme from orange peels

Enzyme extraction from orange peels was applied according to (Okino-Delgado and Fleuri, 2014). Orange peels were cleaned and placed at 4°C for 48 hours. Then it was mechanically crushed at a rate of 0.5 cm^3 and then returned for 48 hours at 4 °C. It was mechanically crushed again for homogeneity. Forty grams of the powder were weighed with the addition of 400 ml of 0.1 M sodium phosphate buffer solution pH 7 in an electric mixer at low speed to avoid shear forces affecting the enzyme. The solution was then filtered with Whiteman

for 30 minutes at a temperature of 4 °C, and the filtrate was taken as a crude extract of the enzyme.

Estimation of enzyme activity

Enzyme activity was measured according to (Chance and Maehly, 1955). We take 10 ml of the tributyrin emulsion in a 100 ml beaker, add 5 ml of buffer solution, and phosphate buffer, add 5 ml of the enzyme solution, and incubate at a temperature of 37 C for 3 hours in a shaking water bath at speed of 60 shakes per minute, then add 30 ml 1 of ethyl alcohol 1 Acetone to stop the reaction, with the addition of a few drops of phenolphthalein, and we carry out the process of denaturation with the base NaOH 0.05 M while preparing the plank under the same conditions while stopping and inhibiting the enzyme by exposing it to the boiling point for 5 minutes.

$$\textbf{Enzyme activity} = \frac{\textit{Volume of the base consumed} * \textit{The standard of the base}}{\textit{Volume of enzyme solution} * \textit{reaction time (min)}} * \mathbf{1000}$$

The unit of activity was defined as the amount of enzyme capable of liberating one microequivalent acid in one minute under estimating conditions.

Measuring protein concentration

The Bradford method was used to determine protein concentration using bovine serum albumin (BSA) as the standard protein (Bradford, 1976; Sahar, 2023).

Purification of lipase enzyme extracted from the intestinal tract of carp fish

Precipitation with ammonium sulphate

The precipitation was done with ammonium sulfate according to (Ali *et al*., 2022b) at saturation ratios (20, 30, 40, 50, 60, 70 and 80%) and under refrigerated conditions. It was slowly added to the enzyme extract while stirring. The centrifugation was then carried out at 10,000 xg for 30 min at 4°C, and then the enzymatic activity of the precipitate and filtrate was measured.

Dialysis

The dialysis process was applied according to the method (Mohamad and Sedrah, 2023) between the enzymatic extract precipitated from the ammonium sulfate step and the buffer solution using bags with a cutoff ranging between 8 and 14 kDa. The process was repeated, replacing the buffer solution, which had a concentration of 0.2 M and a pH 7, every 6 hours, for 24 hours. This process was done at 4 °C. The enzyme was concentrated using polyethylene glycol (PEG), and the enzyme activity was estimated.

Characterization of the enzyme

Determine the optimal pH for enzyme activity

A solution of the substrate was prepared with different pH numbers ranging from 3–4–5 using 0.2 M sodium acetate buffer solution, 0.2 M potassium phosphate buffer solution with a pH of 6–7-8, and 0.2 M Tris-HCL buffer solution with a pH of 9. It incubates the substrate with an enzyme for each at pH 3–9 in test tubes for 30 minutes in a water bath at 37 °C, then stops the reaction by placing the tubes in a water bath at 100 °C for 1 minute and estimates the enzymatic activity (Ali *et al*., 2022b).

Determine the optimal pH for enzyme stability

One ml of purified enzymes was mixed with 1 ml of buffer solutions prepared according to the method above in test tubes and incubated in a water bath at a temperature of 37°C for 30 min. The enzyme activity was estimated using the optimal pH for enzyme activity and the linear relationship between the activities of the enzyme was drawn and different pH values for enzyme activity (Ali *et al*., 2022b).

Determine the optimum temperature for enzyme activity

The optimum temperature for enzyme activity was determined by estimating enzyme activity at different temperatures ranging from 20-80°C at the optimum pH for 30 min (Ali *et al*., 2022b).

Determine the optimal temperature for enzyme stability

The 1 ml of partially purified lipase enzyme solutions was incubated at different temperatures (20, 30, 40, 50, 60, 70 and 80) in test tubes for 30 min. The tubes were transferred to an ice bath. The enzyme activity was estimated at the optimum temperature (Ali *et al*., 2022b).

Results and Discussion

Enzyme extraction

The results showed the enzymatic activity of the lipase enzyme extracted from different sources. It reached 4.122 units/g for the enzyme extracted from the chicken pancreas. For the intestinal lipase of carp, it was 6.532 units g^{-1}, while the vegetable lipase of orange peels was 1.333 units g^{-1}, the sprouted melon seeds were 0.732 units g^{-1}, and the pumpkin seeds were 2.598 units/g (Figure 1). There is a lot of research to compare noticeable superiority of animal sources at the expense of plant sources, which is expected since the digestive system of different animals is a fertile environment for the presence of the lipase enzyme. Therefore, the seeds germinated in an attempt to stimulate them to secrete the enzyme and activate it, as well as by choosing orange peels that could be a competing source for this enzyme.

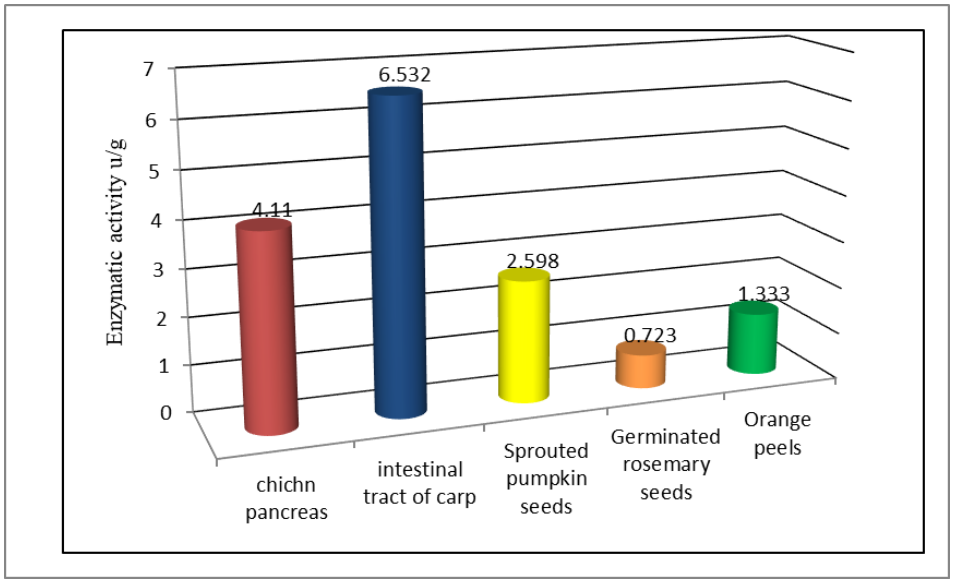

Figure 1. Enzymatic activity of lipase extracted from different sources

The results of enzymatic activity show that the intestinal source of carp fish is the most effective among other sources. It is likely to outperform plant sources, even if they are sprouted. However, in the chicken pancreas, likely, the method of extracting the enzyme using a pestle instead of an electric mixer was chosen in order to avoid possible shearing forces that directly affect the enzyme and are not compatible with the pancreatic tissue. This is the reason behind this increase, in addition to the fact that the intestinal tract of carp is the main place for the enzyme to work in the digestive tract (Ali *et al*., 2022b).

Precipitation with ammonium sulphate

The enzyme extract of lipase enzyme was extracted from the intestinal tract of carp by the salting-out method using solid ammonium sulfate at saturation ratios (20, 30, 40, 50, 60, 70 and 80). The best

percentage was 80% to obtain the lipase enzyme extracted from the intestinal tract of carp, which gave an enzyme activity of 6.664 units g^{-1} (Figure 2).

Then, the dialysis process was mentioned above. The enzyme activity was 6.144 units mL^{-1}. The purified enzyme was partially lyophilized with a lyophilization device to preserve the enzyme. The results show the purification stages of the lipase enzyme in the intestinal tract of common carp. The enzymatic activity reached 1.655 units mL^{-1}, while the specific activity was 0.418 units/mg. After precipitation with 80% ammonium sulfate, the enzymatic activity was 6.314 units mL^{-1} and the activity was 0.664 units mg^{-1} (Table 1). Ali *et al*. (2022b) found that when using the digestive tract of the common carp *L. Carpio Cyprinus*, using a potassium phosphate buffer solution of pH 7, M 0.2, and then the crude extract was precipitated with a saturation rate between 40-80% ammonium sulphate.

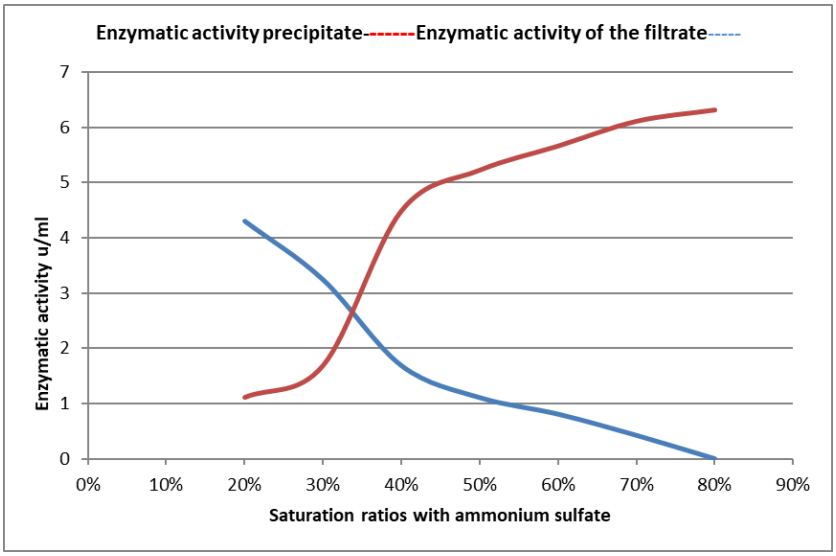

Figure 2. Enzymatic activity of the precipitate and filtrate of the lipase enzyme extracted from the intestinal tract of carp

Kameshwar Sharma *et al.* (2014) reported that the lipase enzyme was extracted and isolated from the digestive tract and intestines of large Indian carp *Catla catla*, and the resulting crude extract was precipitated using ammonium sulphate (20-80%).

After performing the dialysis process, the enzymatic activity was 6.144 units mL^{-1}, and the specific activity recorded 0.685 units mg^{-1}. The number of purification times was 1.638 times, with an enzymatic recorded of 80.180%. It is noted that the enzymatic activity and specific activity increased and the enzymatic activity decreased with the continuation of the purification steps.

Table 1. Purification steps for the purification of lipase enzyme extracted from the intestinal tract of carp

Purification steps	**Vol. of extract** (ml)	**Activity** (u mL^{-1})	**Total activity** (unit)	**Protein con.** (mg mL^{-1})	**Specific activity** (unit mg^{-1})	**Purification fold**	**yield %**
Raw	1250	1.655	2068.75	3.954	0.418	1	100

Precipitation with ammonium sulphate	290	6.314	1831.060	4.194	0.664	1.588	88.510
Dialysis	270	6.144	1658.880	4.212	0.685	1.638	80.180

Enzyme characterization

Determine the optimal pH for enzyme activity

The effect of pH on the activity of the lipase enzyme extracted from the intestinal tract of carp fish was studied at pH (3 and 9). The results found that the optimal pH for the activity of the lipase enzyme extracted from the intestinal tract of carp fish was 7, as the enzyme activity reached 96.82 units g^{-1}.

Figure (3) also shows a decrease in the enzymatic activity of lipase at a pH of 3–9. The enzymatic activity of the lipase at a pH of 4–8 shows great activity. It can be said that the lipase enzyme extracted from the intestinal tract of fish belongs to the group of neutral lipases. This decrease is due to the ionic nature of the enzyme, which affects its activity due to the shape change of the enzyme with these extreme numbers that also lead to a decrease in activity (Price and Stevens, 1988). The best activity was at pH 7. Iqbal and Rehman (2015) indicated that the optimal pH for the activity of the lipase extracted from Bacillus subtilis was 7. As well Kurtovic *et al*. (2010) found the same results. That is, the optimal pH for the lipase of Chinook salmon was 8.5. It was agreed with Ali *et al*. (2021a) for the extracted lipase from *Pseudomonas aeruginosa*.

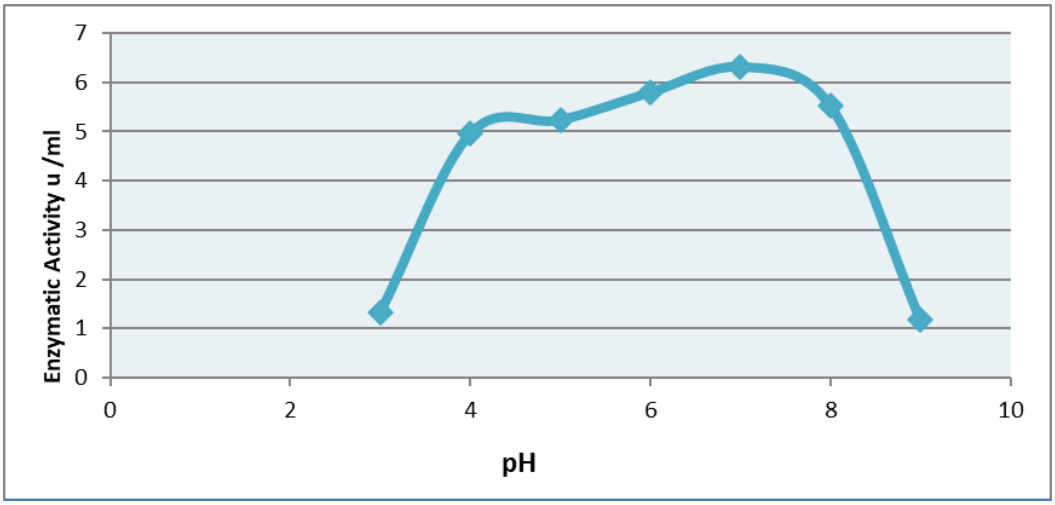

Figure 3. Estimate of the optimal pH for the activity of the enzyme lipase extracted from the intestinal tract of carp

Determine the optimal pH for enzyme stability

The optimum pH for the stability of the lipase enzyme extracted from the intestinal tract of carp fish was determined after incubating it for 30 minutes in buffer solutions with pH numbers 3–9 (Figure 4). It is clear that the lipase enzyme showed stability at pH values of 8–6. It maintained 90–100% of its total activity and was close to (Ali *et al.*, 2022b). In addition, the previous study by Kurtovic *et al.* (2009) confirmed that Chinook salmon *Oncorhynchus tshawytscha* had lipase stability at pH values ranged between 5-8.

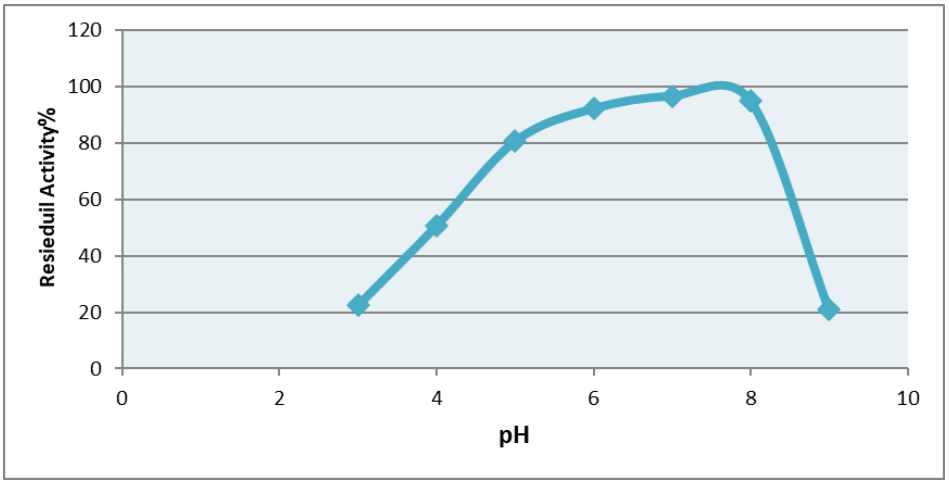

Figure 4.The optimum pH for the stability of the lipase enzyme extracted from the intestinal tract of carp

Determine the optimum temperature for enzyme activity

The effect of temperature on the activity of the lipase enzyme extracted from the intestinal tract of carp fish was estimated by estimating the enzyme activity at temperatures between 20 and 80 °C and at the optimum pH of 7. The results indicate that enzyme activity increases with increasing temperature, reaching its maximum value, which was 96.22 units g^{-1} at a temperature of 40 °C, which represents the optimal temperature for activity. Then, it begins to decrease with increasing temperature (Figure 5).

The increase in activity at 40 °C is due to the increase in energy of the enzyme and substrate, which increases the possibility of collision. Also, the enzyme activity decreases with increasing temperatures due to a change in the conformation of the enzyme or the substrate that may not be subject to the enzyme. Thus, the enzyme cannot function normally, so its activity decreases, especially above 60 °C, and it almost loses its activity at 80 °C. This is because the enzyme lost its natural properties. It was similar to what they found (Ali *et al.*, 2022b). Another study also found that the optimum temperature for lipase *Klebsiella pneumonia* was 30 °C (Emmanuel *et al*., 2020). Indicate that the optimum temperature for *late calcarifer* was 50 °C (Sae-Leaw and Benjakul, 2018). It was also mentioned that the optimum temperature for lipase extracted from *Pseudomonas aeruginosa* was 37 °C (Ali *et al*., 2021b).

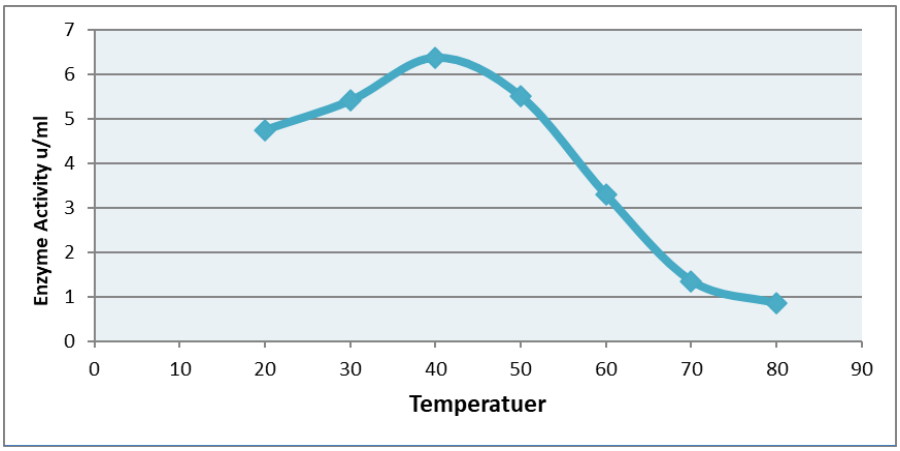

Figure 5. The optimum temperature for the activity of the lipase enzyme extracted from the intestinal tract of carp

Determine the optimum temperature for enzyme stability

The enzyme has been incubated at different temperatures, ranging from 20 to 80 °C for 30 minutes. The results showed that the lipase enzyme extracted from the intestinal tract of carp fish retained its activity after incubation at 20–50 °C. The enzymatic activity of the lipase enzyme extracted from the intestinal tract of carp fish has a wide range of stability, which gives it importance as one of the important enzymes in the food industry. At a temperature of 70, the activity decreases as a result of the change in the shape of the enzyme and the substrate that was affected by the high temperature (Figure 6). The results were similar to those of Ali *et al*. (2022b) and Kameshwar *et al*. (2014), which found that the lipase enzyme extracted from the large Indian carp *Catla catla* (Catla) was stable at temperatures ranging from 30 to 60 °C.

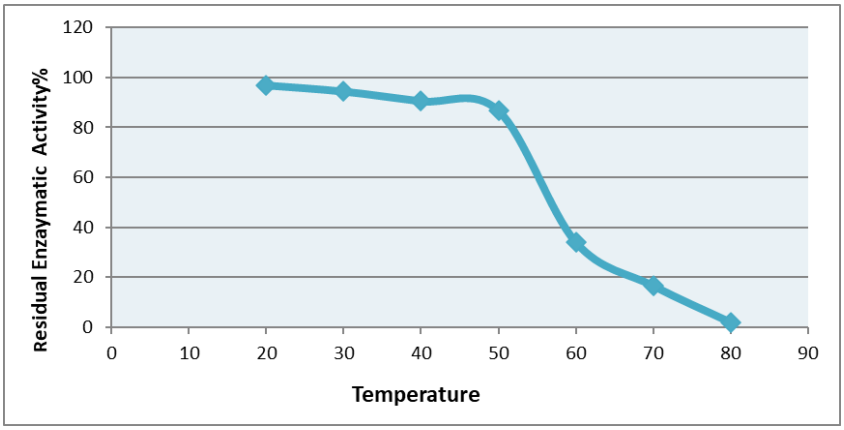

Figure 6. The optimum temperature for the stability of the lipase enzyme extracted from the intestinal tract of carp

Conclusions

The study concludes that the intestinal tract of the common local carp *Carpio Cyprinus* is a rich source of lipase. Potassium phosphate buffer solution was the appropriate solution for extracting lipase from local carp. The intestinal tissue is a fertile place for the enzyme to be present, and precipitation with ammonium sulfate at 40–80% saturation was the best option to concentrate the raw extract. The dialysis and lyophilization contributed to concentrating the enzyme and maintaining its activity. It can also be noted that the enzyme produced from the intestinal tract of common carp can be used in different industrial fields. Therefore, it is able to benefit from sources that may be of little value and from simple extraction methods to produce a product of great value.

Conflict of interest

Regarding the publication of this manuscript, the authors declare that there are no conflicts of interest.

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