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Comparative Study of Different Methods of Extraction of Lipase from Seaweeds.

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ABSTRACT

Lipases are one of the widely used groups of enzymes in food, detergent, cosmetics, organic solvents and pharmaceutical industry as they catalyse the hydrolysis of the triacylglycerols into its simpler compounds. Lipases are involved in the process of transesterification which helps in the production of bio-fuel. These enzymes are having an increasing impact on bioprocessing industry. Here an attempt has been made to extract the enzyme from various varieties of seaweeds from the coastal area of Covelong and Pulicat Lake, Chennai, Tamilnadu. The protein precipitation was compared using three methods and the partial purification of lipase from species such as *Enteromorpha intestinalis, Enteromorpha clathrata, Gracilaria edulis, and Ulva lactuca* was carried out by size exclusion chromatography. The protein estimation showed highest concentration in *E. intestinalis* (117µg/ml) and lowest in *U.lactuca* (87µg/ml). *E.intestinalis* showed the highest lipase activity among all i.e. 0.123meq/min/g with castor oil as a substrate. As these samples are available in bulk quantities nearby coastal area so they were selected for this process as a source of enzymes.

Keywords: Lipases, seaweeds, transesterification, partial purification.

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INTRODUCTION

Seaweeds are algae that live in sea or in brackish water. They are often been called "benthic marine algae", which just means attached algae that live in the sea. Three groups of seaweeds are recognized, according to their pigments that absorb light of particular wavelengths and give them characteristic colour of green, brown or red [1-3]. Because they need light to survive, seaweeds are found only in the relatively shallow parts of the oceans, which means around the shore. Seaweeds come in three basic colours:-red, green and brown. Red and brown are almost exclusively marine, while green algae are also common in fresh water and in terrestrial situations.

Lipase, a subclass of esterases is any of a group of fat-splitting enzymes found in the blood, gastric juices, pancreatic secretions, intestinal juices, and adipose tissues. Lipases have the ability of hydrolyzing fats in the digestive tract but also act as a biocatalyst in carrying out acylation and deacylation of many unnatural substrates. [4-6].

Esterases which can hydrolyze triglycerides at the water/oil boundary are termed lipases or, more systematically, triacylglycerol hydrolases [EC 3.1.1.3]. Lipases are stable in the terms of reactivity and surrounding environment and are obtained from plants, animals and different microbial flora in good yields [7, 8]. From enzyme engineering point of view lipases have a tertiary structure [9]. It exposes catalytically active site in the presence of organic solvents and lipid phase. These enzymes are industrially optimized for the greater use.

In this study an attempt has been made to extract lipase from various varieties of seaweeds such as *Enteromorpha intestinalis, Enteromorpha clathrata, Gracilaria edulis* and *Ulva lactuca* using three extraction procedures namely, ammonium sulphate precipitation, two phase extraction using PEG-4000 and acetone-ether protein precipitation methods [10-13]. The enzyme activity was estimated and compared using two substrates namely, sunflower oil and castor oil.

MATERIALS AND METHODS

Collection of sample

Samples were collected from two different places: Covelong beach and Pulicat Lake.

Identification of sample

The samples were taken to Department of Marine Studies and Coastal Resource Management, MCC, Tambaram, Chennai. The samples were identified by Dr. Lita Sundar.

The samples from Covelong beach was identified as *Enteromorpha intestinalis* and the samples from Pulicat lake were identified as *E.intestinalis, E.clathrata, Ulva lactuca* and *Gracilaria edulis*.

Processing of sample

The samples (macro algae) were washed with water thoroughly several times. After that they were kept under shed for drying for the period of 20 days. The moisture was removed from the sample. The sample was fragmented and was run through mixer-grinder for obtaining powdered form. The powdered sample was kept in the vessel and stored.

Estimation of protein content by Bradford Method

About 5 ml of Bradford reagent was taken in test tube and 1 ml of sample was added. The colour was changed to dark blue. The absorbance was taken at 590 nm in colorimeter [14].

Protein precipitation using Ammonium sulphate

The samples were centrifuged at 10,000 rpm for 10 min. To the clear supernatant 70% ammonium sulphate was added for precipitation [15].

Two phase extraction using PEG-4000

The samples were centrifuged and the supernatant was obtained. An aliquot of 10ml sample was taken and 0.75 g of sodium sulphate was added to it. About 3ml of 50% PEG-4000 was added to check for the formation of two phases. The contents were allowed to settle and the two phases were pipette out using micropipette [16].

Acetone-ether protein precipitation

About 2ml of castor oil and sunflower oil were taken and was neutralized to pH 7.0, with continuous stirring with 25ml of sterile distilled water in the presence of 100mg bile salts till an emulsion was formed. Addition of 2g gum acacia hastened emulsification.

A known quantity of sample (5g) was ground with mortar and pestle or mixer grinder. The tissue was homogenized with twice the volume of ice-cold acetone. The powdered sample was filtered and washed with sterile water, and later successively with acetone, acetone: ether (1:1) and ether. The powder was air dried or placed in a Hot Air Oven for 15-20mins at 40-45°C so that the sample gets dry. The sample was kept in room temperature till it gets back to normal temperature. The powdered sample was refrigerated for the long term use. About 1g of powder was mixed with 20ml ice-cold water and centrifuged at 15,000rpm for 10min and the supernatant was used as an enzyme source [17].

Partial purification by Sephadex G-25 column

Sephadex G-25 was used to pack the column. The solution was fed to sephadex column for desalting and for partial purification.

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Plate Assay

Agar plates were prepared using LB agar containing 1% (v/v) Tween20 or Tween80. The presence of white clear crystal around the enzyme well conferred the presence of lipase activity [18-20].

Lipase Activity meq/min/g sample

The lipase activity was estimated using the following formula.

(vol of alkali consumed * strength of alkali)/ (Wt. of sample in g * time).

RESULTS AND DISCUSSION

In this study, four different species namely, *Enteromorpha clathrata, Enteromorpha intestinalis, Ulva lactuca, Gracilaria edulis* were collected from Covelong and Pulikat Lake for isolating the lipase enzyme using three different extraction procedures and comparing the activity using two different substrates. The protein contents of the crude extract and after ammonium sulphate precipitation are listed in Table 1.

Table 1: Protein concentration of crude samples

Sample	Protein content (µg/ml)	
	Crude extract	Partially purified sample
E. intestinalis (Pulicat)	169	112
E. intestinalis (Kovalam)	179	117
E. clathrata	148	92
U. lactuca	128	87
G. edulis	179	102

Aqueous two phase extraction: Binodal curve

Table 2: Titration of PEG Vs sodium sulphate

Serial No.	% PEG	% Na_2SO4	Volume of PEG (ml)	Volume of Na_2SO4 (ml)	Total Volume (ml)	Weight of PEG (g)	Weight of Na_2SO4 (g)
1.	10%	25%	5ml	2.5	7.5	0.5	0.723
2.	20%	25%	5ml	1.9	6.9	1.0	0.475
3.	30%	25%	5ml	1.6	6.6	1.5	0.400
4.	40%	25%	5ml	1.4	6.4	2.0	0.35
5.	50%	25%	5ml	1.3	6.3	2.5	0.325

The results of the titration of PEG-4000 against sodium sulphate are shown in Table 2. From the results we can conclude that 50% of PEG will be ideal for the protease precipitation. After ammonium sulphate precipitation, the extraction was carried out with PEG-4000. Binodal curve was plotted for knowing the amount of PEG-4000 to be used for extraction. 50%

concentration of PEG-4000 was used and it consumed least amount of sodium sulphate to make solution turbid during titration.

Precipitation using PEG-4000 and citrate buffer

In the process of extraction two different *E.intestinalis* samples were tested from Pulicat Lake and Covelong beach. The sample was pipette out from aqueous phase and it showed nearly similar optical density and concentration when compared with each other.

Lipase was extracted from only two algal samples using the two phase separation method namely, *E.intestinalis* (Pulicat) and *E.intestinalis* (Kovalam) as they showed a significantly higher protein content when compared to other samples. The protein content after precipitation with PEG-4000 is shown in Table 3.

Table 3: Protein precipitation using PEG-4000

Samples	Protein content (µg/ml)
E.intestinalis (Pulicat)	132
E.intestinalis (Kovalam)	127

Lipase activity: Ammonium sulphate precipitation

Table 4- Lipase activity using ammonium sulphate and sunflower oil

Serial No.	Name of sample	Initial pH	After addition of enzyme	Volume of NaOH (ml)	Time (min)	Final pH	Wt of pellet (g)	Activity meq/min/g
1.	*E.intestinalis* (p)	7.02	3.6	10.5	12.52	7.20	0.73	0.115
2.	*E.intestinalis* (k)	7.10	3.58	10.9	13	7.00	0.68	0.122
3.	*E.clathrata*	7.06	3.89	9.1	10.85	7.10	0.70	0.119
4.	*U.lactuca*	7.00	3.66	8.9	10.61	7.04	0.69	0.121
5.	*G.edulis*	7.2	3.90	8.1	9.66	7.10	0.79	0.106

Table 5- Lipase activity using ammonium sulphate and castor oil

Serial No.	Name of sample	Initial pH	After addition of enzyme	Volume of NaOH (ml)	Time (min)	Final pH	Wt of pellet (g)	Activity meq/min/g
1.	*E.intestinalis* (p)	7.02	4.25	9.6	11.44	7.00	0.73	0.114
2.	*E.intestinalis* (k)	7.10	4.46	6.86	8.18	7.13	0.68	0.123
3.	*E.clathrata*	7.06	4.32	7.1	8.46	7.04	0.70	0.119
4.	*U.lactuca*	7.00	4.12	7.5	9.94	7.10	0.69	0.109
5.	*G.edulis*	7.2	4.7	6.2	7.39	7.08	0.79	0.106

The presence of lipase was initially detected by plate assay method (data not shown). Lipase activity was performed by titrometric method with different substrates as sunflower oil and castor oil. The lipase activity showed variation when samples from different precipitation methods were tested. The lipase activity after ammonium sulphate precipitation was estimated

using two substrates namely, sunflower oil and castor oil. The results are tabulated in Tables 4 and 5, respectively.

Lipase activity: PEG-4000

The lipase activity after PEG-4000 precipitation was estimated using two substrates namely, sunflower oil and castor oil. The results are tabulated in Tables 6 and 7, respectively.

Table 6- Lipase activity using PEG-4000 and sunflower oil

Serial No.	Name of sample	Initial pH	After addition of enzyme	Volume of NaOH (ml)	Time (min)	Final pH	Wt of pellet (g)	Activity meq/min/g
1.	*E.intestinalis*(p)	7.10	4.12	7.5	10	7.02	0.73	0.102
2.	*E.intestinalis*(k)	7.10	4.16	7.3	9.73	7.07	0.68	0.110

Table 7- Lipase activity using PEG-4000 and castor oil

Serial No.	Name of sample	Initial pH	After addition of enzyme	Volume of NaOH (ml)	Time (min)	Final pH	Wt of pellet (g)	Activity meq/min/g
1.	*E.intestinalis*(p)	7.10	4.36	7.00	9.33	7.00	0.73	0.102
2.	*E.intestinalis*(k)	7.10	4.42	6.9	9.20	7.01	0.68	0.110

Lipase activity: Acetone-ether

The lipase activity after acetone-ether precipitation was estimated using two substrates namely, sunflower oil and castor oil. The results are tabulated in Tables 8 and 9, respectively.

Table 8-Lipase activity using acetone-ether and sunflower oil

Serial No.	Name of sample	Initial pH	After addition of enzyme	Volume of NaOH (ml)	Time (min)	Final pH	Wt of pellet (g)	Activity meq/min/g
1.	*E.intestinalis*(p)	7.10	5.8	2.8	4.2	7.10	0.73	0.09
2.	*E.intestinalis*(k)	7.10	5.3	2.9	4.35	7.00	0.68	0.098

Table 9- Lipase activity using acetone-ether and castor oil

Serial No.	Name of sample	Initial pH	After addition of enzyme	Volume of NaOH (ml)	Time (min)	Final pH	Wt of pellet (g)	Activity meq/min/g
1.	*E.intestinalis*(p)	7.10	5.6	2.9	4.5	7.03	0.73	0.088
2.	*E.intestinalis*(k)	7.10	5.52	3.1	4.80	7.01	0.68	0.095

The crude extracts of *G.edulis and E.intestinalis* showed highest protein concentration when compared to all other species. Its concentration was found out to be 179µg/ml and lowest was shown by *U.lactuca* i.e. 128µg/ml. In another study [19] the concentration of

protein in *Ulva lactuca* was very less compared to other species as observed here. The protein content of the partially purified samples after ammonium sulphate precipitation was also estimated, wherein they showed a highest protein content of 117µg/ml in *E. intestinalis* and a lowest protein content of 87µg/ml in *U.lactuca*.

The results have clearly shown an increased lipase activity using the ammonium sulphate precipitation method which is followed by PEG-4000 precipitation method and acetone-ether precipitation methods. It might be concluded that ammonium sulphate precipitation is a more stable method of protein precipitation. Moreover, the two substrates used in this study did not show any significant difference in enzyme activity.

Among all the samples tested for lipase activity, *E.intestinalis* from Covelong beach showed the highest activity. It was having the highest lipase activity among all i.e. 0.123meq/min/g with castor oil as a substrate.

CONCLUSIONS

As discussed here, lipases are the enzymes with multiple usages [20]. These are becoming increasingly important in high-value applications in the medicinal industry and the production of variety chemicals and drugs. The extraction showed that red algae were having more protein content than the other green algae tested. In the comparative study it was found out that the ammonium sulphate precipitation was the best suited method for extraction.

There was difference in the activity of lipase when the extraction was performed by three different processes; still no change was observed in the activity of *E.intestinalis* collected from Covelong beach, Chennai. The lipase activity was always more when compared to other collected and tested samples.

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