Production and Characterization of Esterase-producing bacterial strain

Project report submitted in partial fulfillment of the requirements for the degree of

M.Sc. BIOTECHNOLOGY

By

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Under the supervision of

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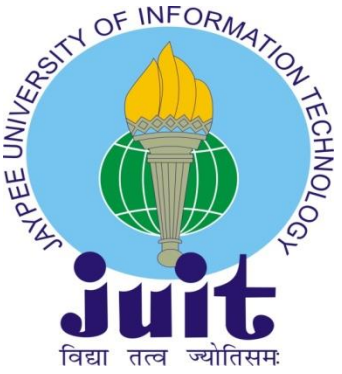

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Declaration

I hereby declare that this thesis entitled “Production and Characterization of Esterase-producing bacterial strain” submitted at Jaypee University of Information Technology, Waknaghat, is an authentic record of my work carried out under the supervision of Dr. Ashok Kumar Nadda and that no part of this thesis has been presented earlier for any degree, diploma, title or recognition.

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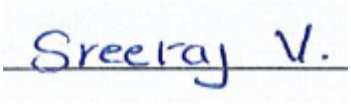

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Supervisor's certificate

This is to certify that the thesis entitled "Production and characterization of Esterase-producing bacterial strain" is an authentic record of work carried out by Sreeraj V, under my supervision in partial fulfillment of the requirements for the M.Sc. degree in Biotechnology of Jaypee University of Information Technology, Solan and further that no part of thereof has been presented before for any other degree.

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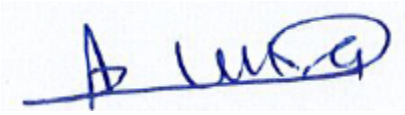

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Contents

Table 1: List of abbreviations

PU	Polyurethane
PHB	Poly(3-hydroxybutyrate)
PET	Polyethylene terephthalate
PVA	Polyvinyl alcohol
PES	Polyethylene succinate
PS	Polystyrene
PUR	Polyester polyurethane
PBSTIL	Poly(butylene succinate/terephthalate/isophthalate)-co-(lactate)
PBST	Poly(butylene succinate-co-terephthalate)
PBSA	Poly(butylene succinate-co-adipate)
PBAT	Poly(butylene adipate-co-terephthalate)

1. Introduction

Esterases (EC 3.1.1.x) are a family of hydrolases that catalyze the synthesis and breakdown of ester bonds. They're found in a wide range of sources including animals, plants, and microbes, and many of them have a wide range of substrate tolerance, implying that they developed to have access to carbon sources or to participate in the catabolic process. Esterases are one of the most commonly utilised biocatalysts since they don't require cofactors, are usually quite stable, and can even function in organic solvents. Esterase activity is often checked using either chromophoric compounds (e.g. p-nitro phenyl esters) or tributyrin-supplemented agar plates. (Bornscheuer ,2002) and the activity can be measured in terms of amount of enzyme that releases 1 μmol of p-nitro phenol from a p-nitrophenyl ester per minute (Sood et al., 2016).

There are four steps in the process of ester hydrolysis or formation: The substrate is first attached to the active serine, resulting in a tetrahedral intermediate supported by the His and Asp residues in the catalytic His and Asp. The alcohol is then released, resulting in the formation of an acyl-enzyme complex. When a nucleophile attacks (water in hydrolysis, alcohol or ester in (trans-) esterification), a second tetrahedral intermediate is formed, which breaks down to give the product (an acid or an ester) and free enzyme (Bornscheuer 2002).

The three-dimensional (3D) structures of enzymes show the characteristic alpha/beta-hydrolase fold (Fig.1). Ser-Asp-His form the catalytic triad. (Bornscheuer 2002). Adjacent to the main alpha/beta-hydrolase fold core domain, these enzymes have a lid domain that covers the active site. The lid domain has an essential function during catalysis: it binds to the hydrophobic substrates, including polyesters, and promotes catalysis by opening the active site, consisting of a highly hydrophobic substrate-binding groove (SBG) (also called channel, pocket, cleft)(Gricajeva et al., 2021).

2. Review of literature

Numerous kinds of lipolytic enzymes are produced by bacteria, including carboxylesterases (EC 3.1.1.1), which hydrolyze tiny ester-containing compounds that are at least partially soluble in water (Arpigny & Jaeger, 1999). The family of lipolytic hydrolase enzymes known as esterases catalyzes the breaking and synthesis of ester bonds in a variety of substrates. Esterase converts an ester into an acid and an alcohol. Esterases can also catalyze the formation of esters and their trans-esterification reactions in addition to hydrolyzing carboxyl ester bonds (Bornscheuer, 2002; Vaquero et al., 2015). Numerous sources, including animals, plants, and microbes, have been reported to produce esterases. Esterases that are sourced from animals show significant difference including physiochemical properties and tissue specific expression between phyla. Esterases from humans and insects are most studied animal esterases due to their significance in human pharmacology and pest control, respectively. Carboxyl esterases, acetylcholine esterases, butrylcholine esterases and cholesterol esterase are used in toxicology and pharmacology studies. Esterases can detoxify insecticides and therefore have an important role in insect physiology. Chlorophyllase, associated with chlorophyll degradation pathways is an earliest known plant esterase (Sharma et al., 2017). Different sources produce different esterases, such as carboxylesterase, cholinesterase, acetyl xylan esterase, aryl esterase, phosphotriesterase, phenolic esterase, pig liver esterase, acetylcholine esterase and tannin esterase (Panda & Gowrishankar, 2004). Bacterial strains are regarded preferable sources of these enzymes than higher species due to the easiness with which they can be mass cultivated and genetically modified. Because of their properties like stability in organic solvents, independent of specific cofactors and broad substrate specificity they are widely used in different industries (Barzakar et al., 2021) including food, pharmaceuticals, detergents, textiles, leather and cosmetics. As a result, extensive efforts are being undertaken to isolate new esterase-producing organisms, as well as to explore the enzymes' characteristics (Dong et al., 2017). Esterases and lipases are both hydrolytic enzymes with certain traits in common but significant differences. True lipase favors water-insoluble substrate (> 6 carbon atoms), whereas esterases prefer simple ester hydrolysis (<6 carbon atoms) (Sharma et al., 2017). p-Nitrophenyl acetate is used for the assay of esterases (Finer et al., 2004). Carboxylesterases have significant catalytic activity against p-nitrophenyl esters throughout a wide pH range of 6–10 (Karaoglu et al., 2011; A. Dukunde et al., 2017).

Previous studies have reported structural information of esterase with the help of crystallographic methods. The basic structure of enzymes is similar, with a classic α/β hydrolase fold (Figure 1) that is seen in superfamilies of hydrolases (Holmquist, 2000). The three dimensional structure of carboxylesterase enzyme is made up of eight stranded β-sheets in the centre, which are surrounded by α-helices and connecting loops (Johan et al., 2021).

Microbial esterase enzymes have two different domains, a catalytic domain and a CAP domain. Two N-terminal helices make up the CAP domain, which is engaged in substrate binding. The active region of the catalytic domain comprises a functional Ser, which is usually found in the consensus sequence G-X-S-X-G. A highly conserved catalytic triad is formed by this Ser, an acidic residue such as Asp or Glu, and a His to which the acidic residue is attached through hydrogen-bond (Barzakar et al., 2021). The catalytic triad creates a charge relay system to generate a nucleophilic serine, positioned in the nucleophilic elbow, that can attack the carbonyl carbon atom of the carboxylic ester substrate. In some unusual cases certain bacterial carboxylesterases have only two catalytic residues, Ser-His, forming a catalytic nucleophile-base dyad (Johan et al., 2021). Another key structural feature that adds to catalytic efficiency is the oxyanion hole (OxH). OxH enhances hydrolysis by forming hydrogen bond between oxygen of carbonyl group of substrate and the enzymes' main chain amides to form OxH that stabilize the intermediate of the hydrolysis process (Gricajeva et al., 2021).

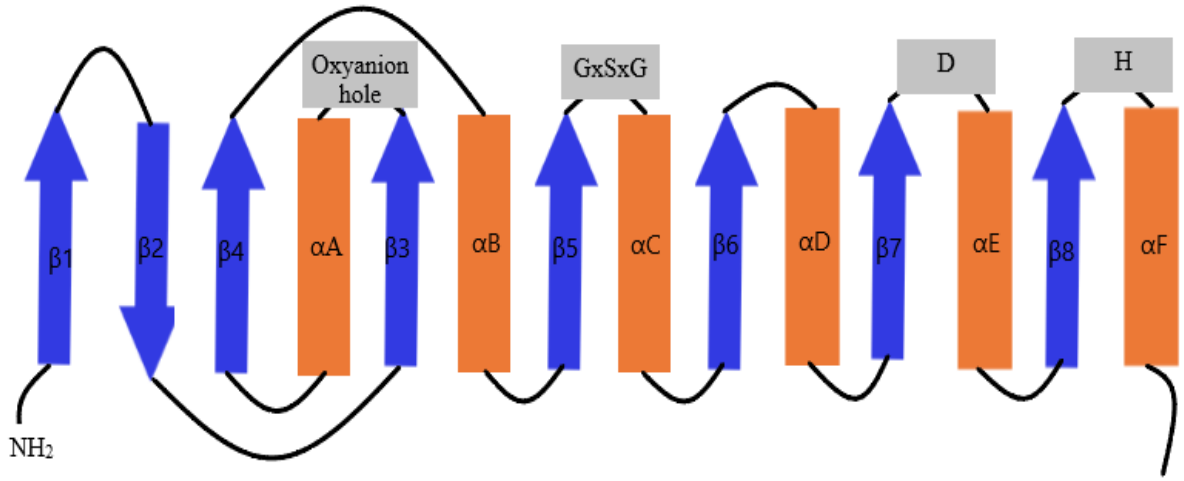

Fig 1: α/β hydrolase fold. β1-β2 and αA-αF indicate β-sheets and α-helices respectively. Positions of histidine (H) and aspartate (D) residues, GxSxG and Oxyanion hole are also indicated. ()

The mechanism of action of ester hydrolysis occur in four steps: I nucleophilic attack of the substrate by active-site serine, to from a tetrahedral intermediate ii) formation of acylated enzyme with related alcohol moiety, iii) attack of the nucleophile with release of second tetrahedral intermediate, and iv) the intermediate breaks down to yield the product and free enzyme (Aranda et al., 2014).

Based on the substrate specificity, inhibition pattern and sequence alignments, different classification systems are given for esterases. The comparison of amino acid sequences can offer a better understanding of the evolutionary connection between enzymes of various sources but it always cannot relate to the enzyme properties. In 1999, Arpigny and Jaeger gave a classification system for bacterial lipolytic enzymes that divided them into eight families based on their amino acid sequences and few biological features (Table 2).

Table 2 :Classification of bacterial lipolytic enzymes(Arpigny & Jaeger, 1999)

Family	Characteristics
Family I	True lipases, largest family with six subfamilies
Family II	Have a Gly-Asp-Ser-(Leu) [GDS(L)] motif instead of Gly-Xaa-Ser-Xaa-Gly
Family III	Lipases family with classic α/β-hydrolase fold and catalytic triad
Family IV	Sequence similar to mammalian hormone sensitive lipase
Family V	Gly-Leu-Ser-Met-Gly consensus sequence
Family VI	Smallest esterases, Gly-Phe-Ser-Gln-Gly conserved sequence motif
Family VII	Gly-Glu-Ser-Ala-Gly sequence
Family VIII	Ser-Xaa-Xaa-Lys motif conserved

Esterases that are sourced from animals show significant difference including physiochemical properties and tissue specific expression between phyla. Esterases from humans and insects are most studied animal esterases due to their significance in human pharmacology and pest control, respectively. Carboxyl esterases, acetylcholine esterases, butrylcholine esterases and cholesterol esterase are used in toxicology and pharmacology studies. Esterases can detoxify insecticides and therefore have an important role in insect physiology. Chlorophyllase, associated with chlorophyll degradation pathways is an earliest known plant esterase (Sharma et al., 2017)

Esterases have various applications in different industries including food and beverages, cosmetics, pharmaceutical, agricultural and chemical. In food and cosmetics industry esterases are used for producing flavoring and fragrance compounds from fatty acids. In diary industry and beverage production, they are used to transform low value fats and oils to high value ones. Previous studies have reported various esterases that are used to degrade polymer compounds that are used in packaging and textile industries (Table 3). In pharmaceutical industry, they are used in production of chiral drugs. Remains of insecticides from agriculture fields can be toxic to the environment and therefore is degraded using esterases. They are also used in various other chemical industries such as textile, leather, and paper (Panda & Gowrishankar, 2004).

Table 3: Polyester degrading esterase enzymes and their sources

Enzyme/ Protein name	**Source**	**Polymer degraded**	**References**
Esterase	Pestalotiopsismicrospora E2712A	PU	Cregut et al., 2013
	Curvulariasenegalensis	PU	Cregut et al., 2013
	Aspergillustubingensis	PU	Khan et al., 2017
	Thermobifida alba	PET	Wang et al., 2011
	Pseudomonas vesicularis PD	PVA	Wilkes &Aristilde, 2017
	Pseudomonas sp. AKS2	PES	Wilkes &Aristilde, 2017
	Pseudomonas sp.	PS	Wilkes &Aristilde, 2017
	Pseudomonas fluorescens	PUR	Wilkes &Aristilde, 2017
	Pseudomonas aeruginosa	PUR	Wilkes &Aristilde, 2017
	Thermobifida alba Est119	PET	Shah et al., 2014
	Roseatelesdepolymerans TB-87	PBSTIL,PBST, PBSA,PBAT	Shah et al., 2014
	Leptothrix sp. strain TB-71	PES,PCL,PBSTIL, PBST,PBSA,PBS, PBAT	Shah et al., 2014
Polyurethanase esterase A	Pseudomonas chlororaphis	PU	Stern & Howard, 2000
Carboxylesterase	Uncultured microrganism	PBAT	Müller et al., 2017

3. Materials and methods

3.1 Isolation of bacteria from soil samples

Soil sample collected from Waknaghat, Himachal Pradesh (31.0084633, 77.0951877), was serially diluted and spread plated. Individual colonies (Isolate no. 1-7) were then streaked on nutrient agar plates with 3mM p-Nitrophenyl acetate (p-NPA) (Sharma et al.,2016). All 7 isolates were checked for esterase activity. Isolate 3, which showed the highest enzyme activity (0.57 U/ml) among the 7 isolates was used for further studies.

3.2 Production and extraction of enzyme

A seed culture was prepared by inoculating isolate in 50 ml nutrient broth and incubated at 38°C for 24 h at 130 rpm. For production of esterase, 1% (v/v) of seed culture was inoculated in 50ml nutrient broth and incubated at 38°C for 24h at 130rpm. The culture was centrifuged at 8000rpm for 10 min at 4°C. The pellet obtained was resuspended in phosphate buffer (50mM, pH 7.5). Cell lysis was carried out by sonication for 5 min (10 sec pulse). The suspension was centrifuged at 8000rpm for 10 min at 4°C. The supernatant obtained after each centrifugation was used as enzyme samples and checked for esterase activity.

3.3 Esterase assay

Esterase activity was measured by measuring the micromoles of p-nitro phenol (p-NP) released from p-Nitrophenyl acetate (p-NPA). To 825µL of phosphate buffer (50mM, pH 7.5) 175µL of substrate (10mM p-NPA) was added. The reaction mixture was incubated at 37°C in a water bath for 5 min. After the incubation 25µL of enzyme sample was added. The reaction mixture was again incubated at 37°C in a water bath for 5 min. The amount of p-NP released was measured from the absorbance at 410 nm. One unit (U) of esterase activity was defined as the amount of enzyme required to release one micromole of p-NP from the substrate p-NPA per minute. Protein estimation was done by Bradford assay (Bradford, 1976).

Out of the 7 isolates that were checked for the enzyme activity, isolate 3 showed highest activity and was used for further studies.

3.4 Optimization of culture and reaction parameters

Basal salt media, Luria broth, Minimal salt media, Muller Hinton broth, nutrient broth and peptone broth were used for culturing the bacterial strain. Luria broth, Muller Hinton broth and nutrient broth were used as obtained from Hi Media. The composition of other media used were, Basal salt media(g/L) - Sucrose 5.0, $MgSO_4.7H_2O$ 0.5, Na_2HPO_4 2.0, $FeCl_3 \cdot 6H_2O$ 0.005, $CaCO_3$ 0.1; Minimal salt media(g/L) - KH_2PO_4 6.8, $MgSO_4$ 0.2, Na_2HPO_4, 7.8, $ZnCl_2$ 0.02, $ZnSO_4.7H_2O$ 0.05, $NaNO_3$, 0.085 peptone broth(g/L) - beef extract 3.0, glucose 1.0, NaCl 5.0, peptone 5.0, $CaCO_3$ 6.0

To determine the effect of inoculum size, production media was inoculated with 0.25-2.5% (v/v) of I.3 seed culture and incubated at 38°C. The optimal temperature for esterase production was determined by incubating at various temperatures ranging from 20°C to 70°C. Esterase activity for each production media was determined using standard activity assay.

To determine the optimal reaction conditions for esterase activity, effect of reaction time, buffer pH and reaction temperature were studied.

3.5 Biochemical characterization of isolated bacteria

Biochemical characterization was done by gram staining, citrate utilization test, urease test and catalase test procedures.

4. Results & Discussion

4.1 Screening for esterase activity in bacteria

Among the 7 isolates obtained from nutrient agar plates with p-NPA, isolate number 3 (I.3) (Fig. 2) showed highest enzyme activity for cell lysate (0.57 U/ml) (Table 2). The protein concentration of the enzyme sample was estimated using Bradford's assay (13.03µg/mL) and the specific activity was calculated (0.044 U/µg). Isolate 3 was Gram stained and was found to be gram positive rod shaped. Colonies of I.3 were observed to be pale yellow in color and irregularly edged.

Table 3: Showing enzyme activity of all 7 isolates.

Sample No./Strain	**Enzyme activity (U/mL)**	
	Supernatant	**Pellet**
1	0.36	0.42
2	0.23	0.46
3	0.18	0.57
4	0.17	0.33
5	0.21	0.35
6	0.21	0.33
7	0.35	0.56

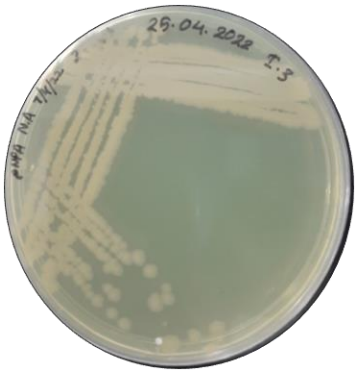

Fig. 2: Isolate No. 3 (I.3) on Nutrient agar plate

4.2 Enzyme production

Among the six different media used, Basal salt media, Luria broth, Minimal salt media, Muller Hinton broth, nutrient broth and peptone broth, enzyme production was observed to be maximum in nutrient broth (Fig.3.a). Maximum protein production was observed for inoculum size of 0.25% (Fig. 3.b). Optimal temperature and pH for enzyme production was found to be 30°C and 8.5 respectively (Fig. 3.c & d).

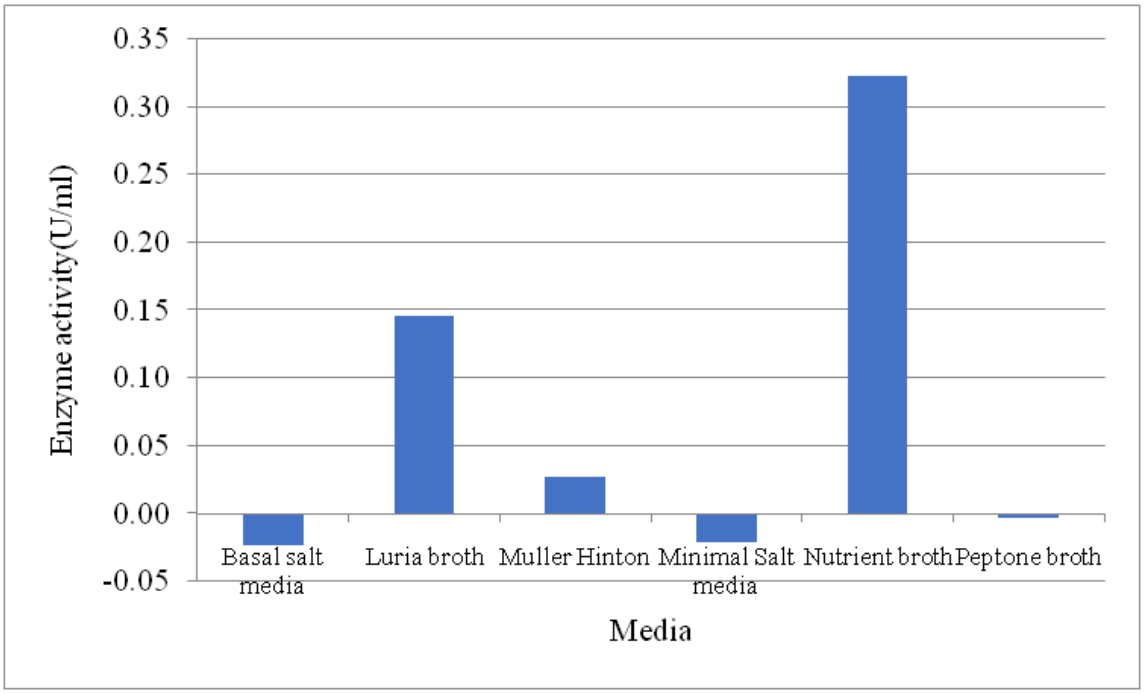

Fig. 3.a: Graph showing enzyme activity for different production media

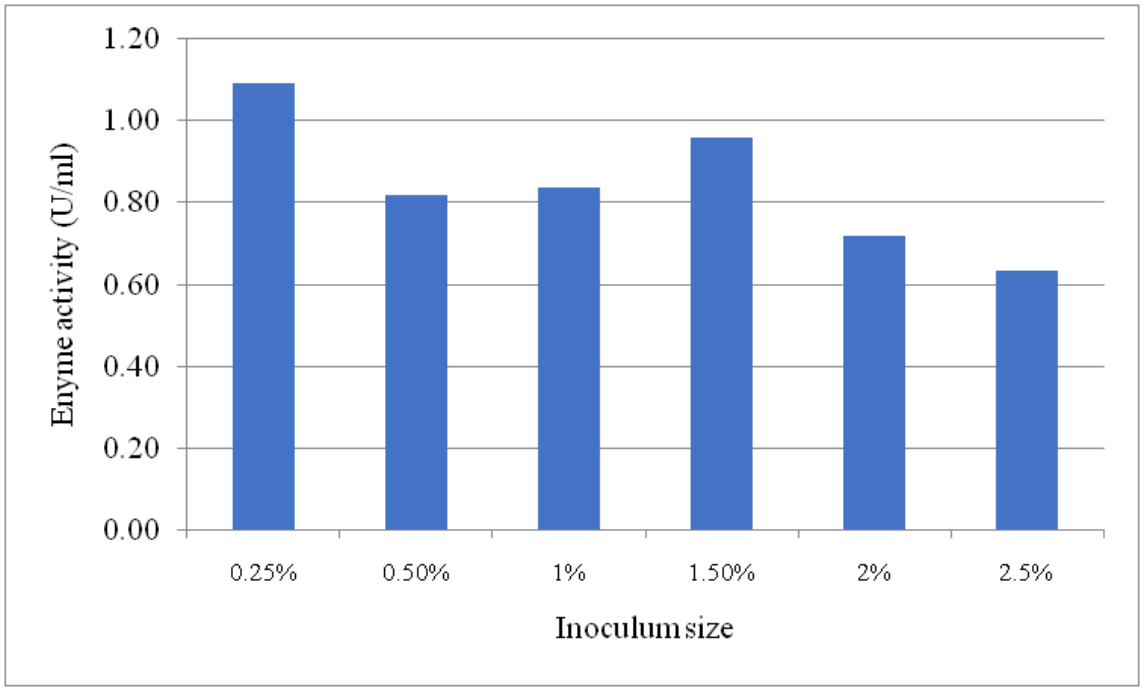

Fig. 3.b: Graph showing enzyme activity for different inoculum size

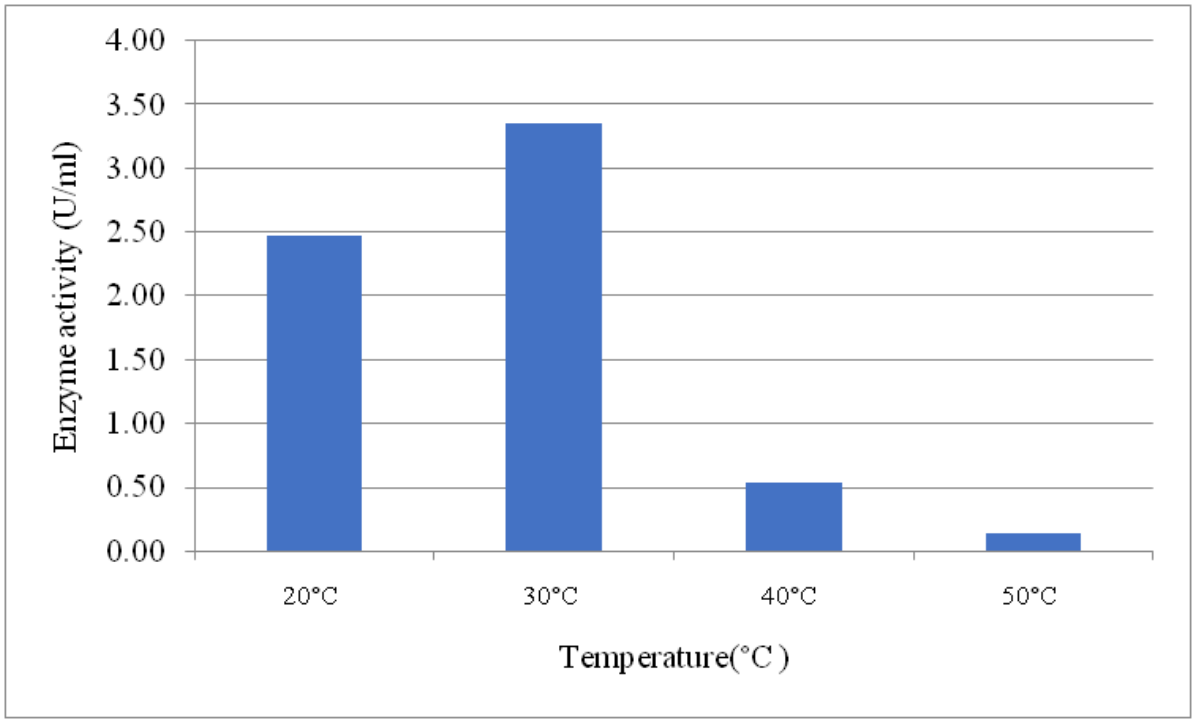

Fig. 3.c: Graph showing enzyme activity for different production temperature

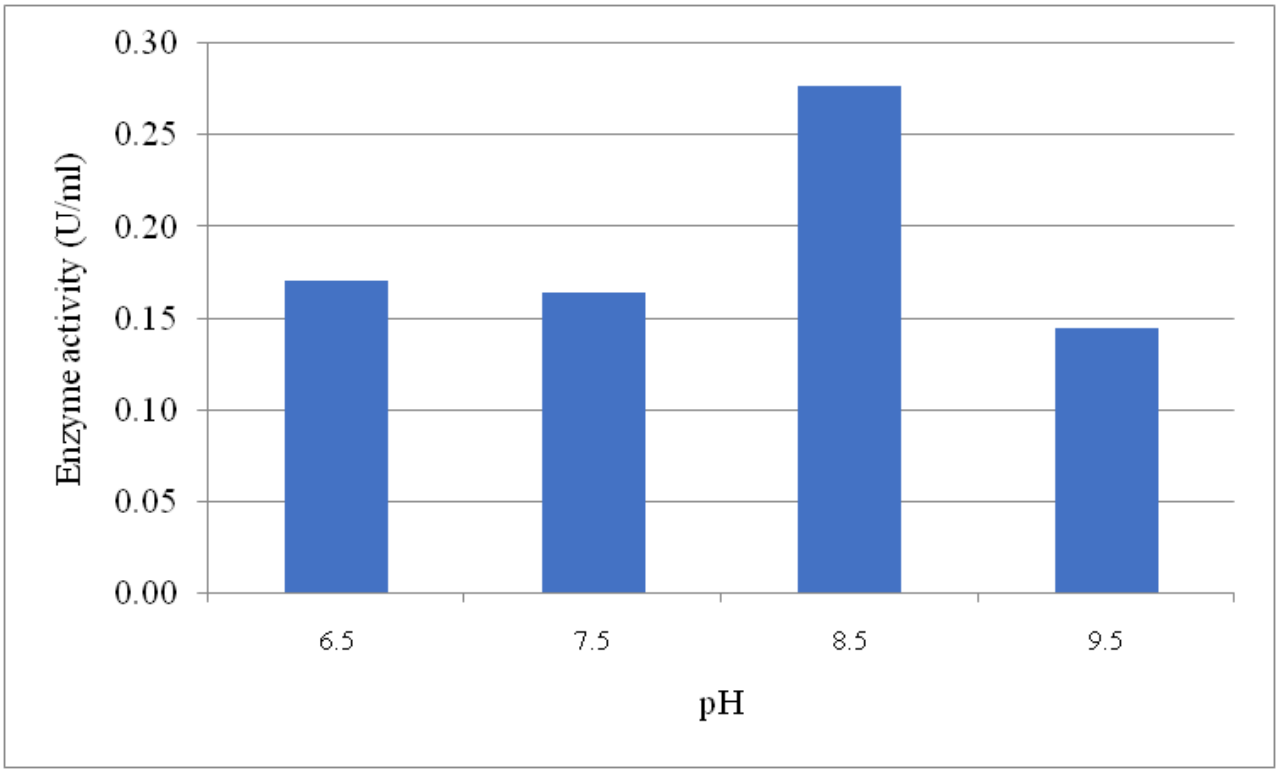

Fig. 3.d: Graph showing enzyme activity for different production pH

4.3 Reaction conditions

Reaction time of 3 to 15 minutes gave similar values after which there was reduction in the enzyme activity (Graph 4.a). The reaction gave maximum yield at pH 9.0 (Graph 4.b). Optimal reaction temperature was found to be 30℃ and reaction was observed to be reduced by 100% at 50 ℃ Graph 4.c).

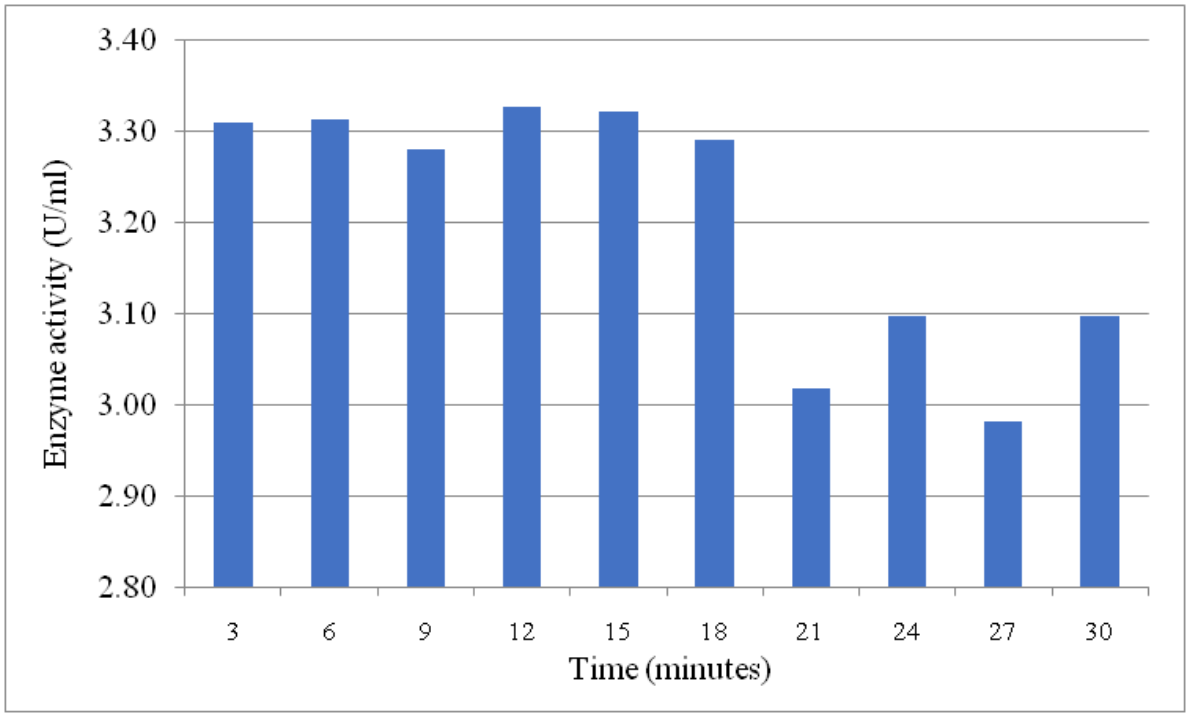

Fig. 4.a: Graph showing enzyme activity for different reaction time

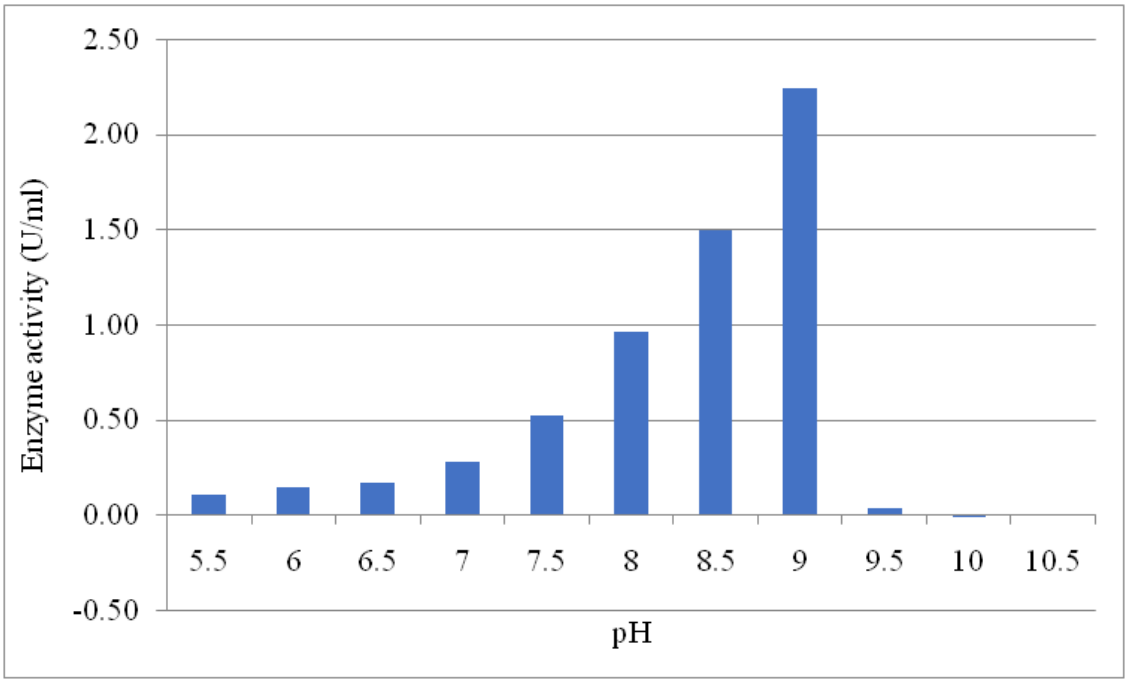

Fig. 4.b: Graph showing enzyme activity for different reaction time

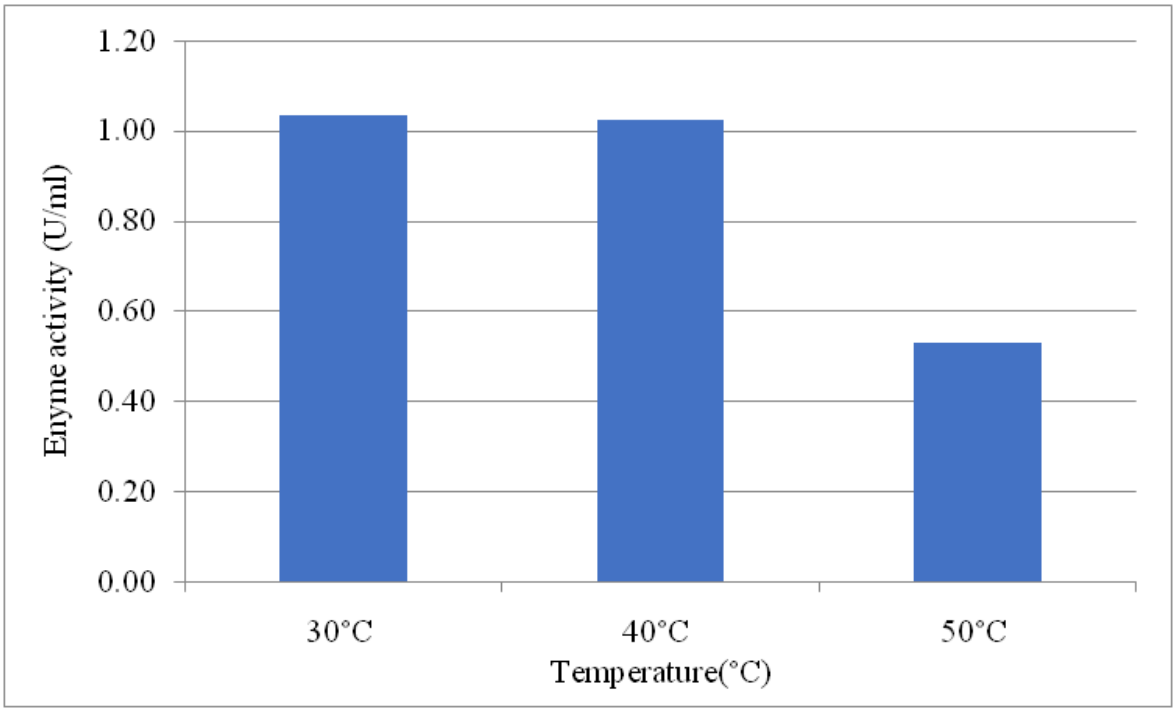

Fig. 4.c: Graph showing enzyme activity for different reaction time

The study involved screening of bacterial strains capable of producing esterase and characterization of production and reaction parameters for esterase. Higher values for enzyme activity were obtained for crude enzyme extract from pellet which indicates that the enzyme was intracellular. The optimum conditions for esterase production are a pH of 8.5, temperature of 30℃, inoculum size of 0.25% in nutrient broth. For the reaction conditions, pH of 9, temperature of 30℃ and reaction time of 3-18 minutes were found to be optimum. Enzyme activity was observed to be low when compared to recent studies (Sharma et al., 2016; Sriyapai et al., 2018) which point out the need for improving selection of sample collection locations.

5. Conclusion

Esterase is one among the most commonly used biocatalysts across various industries. Remediation of pollution caused by polyester compounds is a promising application of esterase enzymes. Bacterial esterases can be comparatively easily isolated and modified and therefore possess great potential to be used in commercial scale. Further studies can be done to explore enzymes with better catalytic efficiency and to improve techniques for isolation, engineering and commercial use of the enzyme.

6. References

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7. Annexure

Enzyme assay for culture/production parameter- Media

Media	**Enzyme activity (U/mL)**
Basal salt media	-0.02
Luria broth	0.15
Muller Hinton	0.03
Minimal Salt media	-0.02
Nutrient broth	0.32
Peptone broth	0.00

Enzyme assay for culture/production parameter- Inoculum size

Inoculum size	**Enzyme activity (U/mL)**
0.25%	1.09
0.50%	0.82
1.00%	0.84
1.50%	0.96
2.00%	0.72
2.50%	0.63

Enzyme assay for culture/production parameter- Temperature

Temperature (°C)	**Enzyme activity (U/mL)**
20	2.48
30	3.35
40	0.54
50	0.14

Enzyme assay for culture/production parameter- pH

pH	**Enzyme activity (U/mL)**
5.5	0.17
6.5	0.16
7.5	0.28
8.5	0.14
9.5	0.17

Enzyme assay for reaction parameter- Time

Time(Minutes)	Enzyme activity (U/mL)
3	3.31
6	3.31
9	3.28
12	3.33
15	3.32
18	3.29
21	3.02
24	3.10
27	2.98
30	3.10

Enzyme assay for reaction parameter- pH

pH	Enzyme activity (U/mL)
5.5	0.10
6.0	0.14
6.5	0.17
7.0	0.28
7.5	0.52
8.0	0.96
8.5	1.49
9.0	2.24
9.5	0.03
10.0	-0.01
10.5	0.00

Enzyme assay for reaction parameter- Temperaturex

Temperature (°C)	**Enzyme activity (U/mL)**
30	1.03
40	1.02
50	0.53

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