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Research Paper

Purification and properties of acetylesterase involved in decolorization of triphenylmethane dyes by *Bacillus* spp.

Patil Sadichha[1], Kulkarni Sayali[1], Patil Rajendra[2], *Satpute Surekha[1]
[1]Dept of Microbiology, Sinhgad College of Science, Ambegaon (BK) Pune - 411041, Maharashtra, INDIA
[2]Dept of Biotechnology, University of Pune, Ganeshkhind, Pune- 411007, Maharashtra, INDIA

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Abstract

Esterases [EC 3.1.1.x.] are the enzymes of hydrolase class involved in catalysis of cleavage and formation of ester bonds. Esterases are involved in interesterification, intraesterification and transesterification reactions. In the present study, different bacterial cultures were isolated from soil, screened for esterase activity and the most potent organism was selected for esterase production. Optimum conditions for esterase enzyme production was found to be at temperature of 37°C, pH – 7, ethyl acetate (1% v/v) as substrate concentration. The esterase was extracted and purified using ammonium sulfate precipitation (80%) and dialysis. Enzyme assay was done using para-nitrophenyl acetate. One unit of enzyme activity was defined as the amount of enzyme liberating 1 µmol of para-nitrophenol per minute. Specific activity was expressed in units per min per mg protein under assay conditions. According to the Lineweaver – Burk Plot, the kinetic properties of esterase were found to be K_{m} = 0.91mM, V_{m} = 213µM/min/mg, K_{cat} = 234/min, Catalytic efficiency = 257.14/mM/min. The molecular weight of esterase enzyme found was 62kDa, possessing decolorizing property triphenylmethane dyes namely malachite green. However, no such activity was observed with azo dye namely Congo red. The industrial applications of esterases provide an immense contribution to the eco-friendly approaches towards nature. We would like to highlight that esterases are less explored enzymes having low literature survey as compared to lipases so the future scope of research is highly valuable. Amongst the number of reports on enzymatic decolourization of synthetic dyes, esterases also provide a significant decolourization potential.

KEYWORDS: Dyes, esterase, ethyl acetate, industrial applications, purification.

Introduction

Esterases are enzymes which belong to the hydrolase family (E.C 3.1.1.x) which are responsible for the breaking and making of ester bond in a chemical reaction with water called hydrolysis. The x in the enzyme commission number depends on the substrate [1]. The hydrolase family consists of two major types of enzymes-lipases and esterases. They both share some properties while possessing some unique properties also. Both the enzymes show a characteristic α/β hydrolase fold in their 3D structure [1,2] and also contain a catalytic triad of Ser-Asp-His [1,3,4]. At the active site, serine is mostly found embedded in the consensus sequence G-X-S-X-G (where X is any amino acid) while other reported motifs include

GDSL, GDXD[5]. Esterases and lipases work at different pH owing to their use in different reactions. The active site of esterases has a negative potential at pH 6.0 while that of lipases is at pH 8.0[1]. Esterases are known to hydrolyze short esters (C≤12) while lipases are capable of hydrolyzing long chain (C≥12) triglycerides[2,4,6]. Esterases do not require cofactors and so they are considered as attractive biocatalysts[7].

There are two possible mechanisms which can be responsible for breaking of ester bond. The first mechanism is similar to a SN2 (bimolecular nucleophilic substitution) where a water molecule directly attacks the ester bond. The second possible mechanism of ester cleavage is a two-step reaction. The first reaction includes the attack on ester bond by a catalytic nucleophile (ionized amino acid chain). The acetyl group is transferred to enzyme nucleophile with simultaneous release of alcohol or amine. This transacetylation reaction may be facilitated by a protonation of ester oxygen which is mediated by a Brönsted acid which helps in cleaving the C-O ester bond. The second reaction cleavage of acetyl-enzyme intermediate takes place. This can be enhanced by a Brönsted base mediated subtraction of protons[8].

Esterase enzymes are used in various industrial applications and have high commercial values. The various applications of the esterases include: Enzymatic degradation of plastic[9], ester synthesis[10], preparation of optically active compounds [11], deinking of printed paper and stickies removal [12], flavouring in cheese[13], release of ferulic acid during wort and beer production [14]. Various aspects namely the screening process, purification protocols, for studying esterase enzyme has been discussed recently[15]. A highlight on potential applications of esterase enzymes in diverse fields is also discussed. Authors also concluded that esterases are less explored enzymes having low literature survey as compared to lipases. Therefore, the future scope on esterase enzyme is highly valuable. The industrial applications of esterases provide an immense contribution to the eco-friendly approaches towards nature as also in the food, textile industries.

Materials and Methods

Soil sample collection, isolation and screening of bacterial cultures for esterase activity

Five soil samples were collected from the Sinhgad college campus, Ambegaon (BK) Pune-411041, Maharashtra, India. All soil samples were serially diluted with sterile distilled water and spread on the nutrient agar plates followed by incubation for 24-48 h at 37 °C for the growth of bacterial cultures. After appearance of microbial colonies on nutrient agar plates, they were purified and subjected to qualitative screening for detection of esterase enzyme producing bacterial isolates. Various media like tributyrin agar (TBA), tween 20 and tween 80 agar plates were used for detection process. In addition to these media, screening of the isolated bacterial cultures for esterase activity was done by Plate Assay method. For this assay, Luria-Bertani agar plates with 1% substrate (ethyl acetate) were prepared & 18 bacterial cultures (SCOS1-18) were tested for esterase production. The bacterial cultures were spot inoculated with the sterile tooth prick and plates were incubated overnight at 37°C. The enzyme activity was checked by spreading Lugol's iodine solution (2% iodine and 4% potassium iodide in water) [16]. This is leading to a starch-iodine reaction indicating zones of hydrolysis. The clear area in the medium around the colony indicated esterase enzyme production[2,4,5]. The most potent organism (SCOS 07) was then characterized and identified using Bergey's Manual of Determinative Bacteriology (9th edition) Details of morphological & biochemical characters are listed in Table 1.

Optimization of environmental parameters for fermentation: Several environmental parameters show significant impact on optimization of any fermentation process. Following were the environmental parameters checked [15,17].

Temperature: the ability of the organism to produce esterase at various temperatures [30°C, 37°C, 45°C, 55°C, and 65°C] was tested.

pH: the ability of the organism to produce esterase at various pH [3, 4, 5, 6, 7, 8, 9,10] was tested. The pH of the media was adjusted using 1M HCl or 1M NaOH.

Substrate concentration: Ethyl acetate was chosen as the substrate and various concentrations like 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5% were tested.

Salts: the effect of various salts – stimulatory or inhibitory- was checked by inoculating the organisms into media containing 10 mM of $MgSO_4$, $CaSO_4$, $CuSO_4$, $MnSO_4$, $ZnSO_4$, $CaCl_2$, $HgCl_2$, NaCl.

Table 1: Morphological and Biochemical characteristics

Character	Observation
Size	2 mm
Shape	Round
Colour	Cream
Margin	Entire
Elevation	Flat
Consistency	Dry
Opacity	Opaque
Gram Character	Gram Positive rods
Motility	Motile
Catalase	Positive
Starch hydrolysis	Positive
Sugar fermentation	Positive

Determination of nature of esterase

Enzyme production by microbial cultures can be constitutive or inducible. Constitutive enzymes are produced constitutively by the cell under all physiological conditions. Inducible enzymes are normally present in minute quantities within a cell, but whose concentration increases dramatically when a substrate compound is added. In the present studies, the nature of the enzyme production (constitutive or inducible) was checked by inoculating culture in nutrient broth with and without the substrate (ethyl acetate) and incubated overnight at 37°C. The esterase activity of both was calculated [15,16].

Determination of location of esterase

To determine the location of enzyme (i.e. whether the enzyme is extracellular, intracellular or membrane-bound) three cell fractions were prepared. The extracellular fraction was the supernatant obtained after centrifuging the culture broth. The pellet obtained was resuspended in the culture medium and it was lysed using SDS. It was then centrifuged and the supernatant was kept as the intracellular fraction. The pellet obtained was further resuspended in the culture medium and considered as the membrane- bound fraction. The enzyme activity of each fraction was estimated. Highest enzyme activity is considered to indicate the location of enzyme [15,16].

Relation between growth curve of the organism (SCOS07) and esterase formation

There is a relation between growth curve of the organism and product formation. The maximum product formation is at a particular phase in the growth curve. To study this, the organism was inoculated in nutrient medium supplemented with 1% ethyl acetate and incubated at 37°C. At every hour, the absorbance was taken at 400 nm on colorimeter. At 1hour interval, 1ml of the inoculated medium was removed in sterile microfuge tubes and stored in refrigerator. The esterase activity at each hour was estimated and it was correlated with the growth phase of organism[18].

Lab-scale fermentation for esterase production

Fermentation was carried out in 500 ml Erlenmeyer flask containing 100 ml of LB broth supplemented with 1% ethyl acetate. About 1% of overnight fresh grown culture was inoculated and the flask was incubated on rotary shaker at low speed (300 rpm) and the product was harvested after four days[19,20,21,22].

Extraction and purification of crude enzyme from fermentation broth

The fermented broth was centrifuged for 10000 rpm at 4°C for 10 minutes. The pellet was discarded and the supernatant was kept as the crude enzyme. Partial purification of the crude enzyme was done using following two methods.

Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is a commonly used method to purify enzymes[15]. The method is based on the salting out process. Ammonium sulfate is frequently used due to its high solubility in water, it has no adverse effect on enzyme activity as well as it is cheap. The protocol included addition of increased ammonium sulfate concentration in the crude enzyme. Precipitation was obtained after 24 hrs of refrigeration. To get a better yield, the mixture was rotated on a magnetic stirrer at low speed, overnight at 4°C.

Dialysis

This is a method of protein purification where semi-permeable membranes are used. Himedia Dialysis membrane -150, Aveage flat width- 42.44 mm, Av diameter -25.4 mm, Capacity approximately – 5.07 ml/cm was used. The pore size of these membranes is such that small molecules like salt ions can pass easily whereas the large molecules like proteins remain in the membrane itself. The molecular cut off of the dialysis membrane is an important consideration for purifying the protein of interest. The different membranes which can be used for dialysis are made from regenerated cellulose or cellophane. Cut the dialysis tubing (approx 7 cm in length) and heat the dialysis bag at 95°C for 10 minutes. Further, dialysis tubing was washed with distilled water. The process was repeated for three times. The dialysis tubing was tied from one end using thread The ammonium sulfate precipitated enzyme was filled from the open end and tubing was tied carefully from other side without keeping any air bubbles This dialysis tubing was then placed in 200-500 times sodium phosphate buffer (pH7.0) than the sample at room temperature. Buffer was changed after 2hrs and kept again at room temperature. Dialyzed esterase enzyme was obtained.

Enzyme assay

Quantitative assay of the enzyme gives the specific units of the enzyme. The reaction mixture contained 1ml of 0.15 M sodium phosphate buffer (pH 7.0) + 0.5 ml of 1 mM p-nitrophenyl acetate + 2.5 ml of distilled water + 0.5 ml of enzyme. This was incubated at 30°C for 30mins. Then absorbance was taken at 400nm.One unit of esterase activity is defined as the amount of enzyme producing 1 µM of p-nitrophenol per minute at 30°C [2,6].

Protein estimation by Folin-Lowry method

Folin-Lowry method is one of the most common methods used for protein estimation. It can detect proteins upto 1mg/ml concentration. The Folin Ciocalteu reagent is reduced by the side chains of phenolic groups in a protein to give a deep blue color. Bovine serum albumin was used as the standard [2,6,18-21]

Molecular weight determination of the esterase enzyme using SDS- PAGE

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis is a technique widely used in determination of molecular weight of proteins. The enzyme sample was run onto 4% stacking gel and 10% resolving gel and stained using the silver staining procedure [2, 4, 6, 8, 18-21].

Determination of kinetic properties of esterase

Enzyme kinetics is the measure of the reaction rate catalyzed by that enzyme and the effects of various conditions. The Michealis Menten constant (km), maximum velocity (Vmax), turnover number (kcat) were determined [2, 3, 6, 23, 27].

Stability studies of esterase

The esterase enzyme was checked for its pH and temperature stability by incubating the enzyme at different pH and temperature[15, 23, 24].

TLC analysis of esterase

Sample preparation– Heat enzyme with 6N HCl (5-50 times the volume of enzyme) at 110°C for 18-24 hrs. Solvent system – Butanol: acetic acid: water (5:4:1), Developer – Ninhydrin solution[25].

To check the potential of esterase in decolorization of dyes

In microbiology laboratories, the triphenylmethane dyes i.e. crystal violet and malachite green are routinely used. These dyes are very harmful to the environment. Decolorization of these dyes is one step in their degradation. The ability of our microbial esterase to decolorize these dyes was tested. 1mg/ml stock solution of the dyes was prepared and 5% esterase was added to it. The change in absorbance after 24hrs was taken and considered as a positive result for decolorization [26].

$$\% \text{ decolorization } = \frac{\text{Initial OD} - \text{ Final OD}}{\text{Initial OD}} \times 100$$

Results and Discussion

Plants, animals and micro-organisms are the common sources for various enzymes. Microbial enzymes are mainly attractive owing to their low cost to grow and maintain them and also due to their ease of handling, availability of different techniques which can be easily used to manipulate them. Bacteria, fungi, actinomycetes produce esterases and their novel species are being explored for obtaining esterases with novel functions. Different microbial sources for esterase production are usually incurred from culture collections or can be isolated. In the current study, the bacteria producing esterase was isolated from soil. There are reports where the organisms were isolated from oil contaminated areas of city garbage [5], from cheese surface [10] for esterase production. A recent approach of metagenome screening for esterase activity is being used [32, 39].

The inducible, extracellular acetyl esterase was extracted from *Bacillus* spp. isolated from soil and qualitatively detected by plate assay using Lugol's iodine to enhance the clear zones. The clear zones around the colonies highlight the ability of esterase to hydrolyse the substrate (ethyl acetate). Total 18 bacterial cultures were studied for esterase enzyme production, out of which, 11 bacterial cultures found to be esterase positive by plate assay method. The most potent esterase producer was selected which showed the maximum halo as indicated in Figure 1. According to the Bergey's Manual of Determinative Bacteriology (9th edition), based on morphological and biochemical characteristics, the isolate SCOS 07 may belong to *Bacillus* spp.

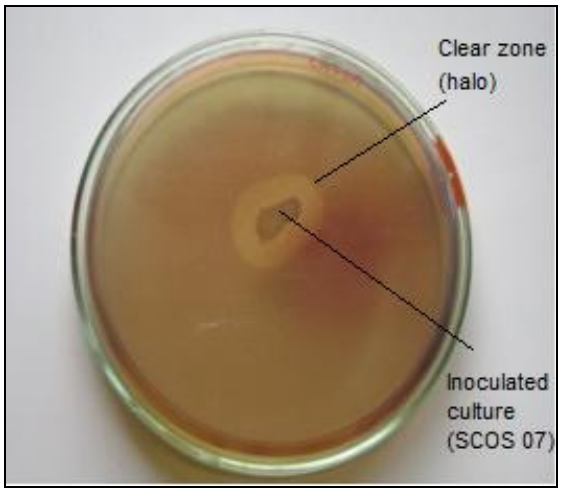

Figure 1: Selection of most potent bacterium (*Bacillus* sp.) on Luria agar medium supplemented with ethyl acetate (1%) for esterase production

Literature survey indicated that number of different substrates have been utilized efficiently for microbial esterase enzyme production. Ethyl acetate is one of the common substrates used for acetyl esterase production. In our current studies, the optimum conditions for submerged fermentation were found to be a substrate concentration of 1% (v/v), Temperature of 37°C at pH 7. Esterase enzyme is an inducible enzyme obtained from *Bacillus* spp. with the substrate ethyl acetate 91%). It was found to be extracellular in nature. Maximum esterase enzyme production was obtained in the stationary phase of *Bacillus* spp. Figure 2 indicates that the maximum product formation corresponds to the stationary phase of *Bacillus.*

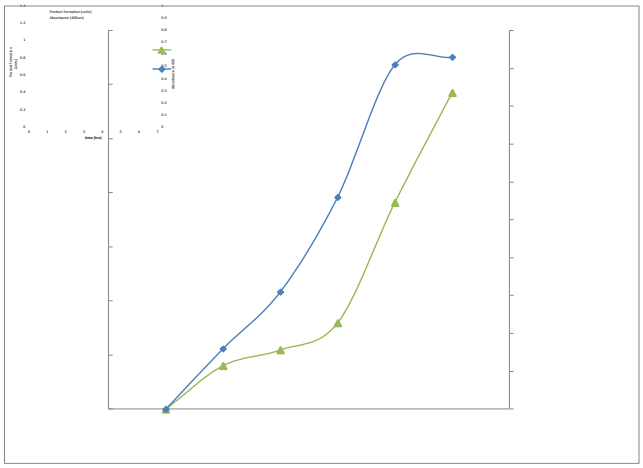

Figure 2: Graph showing the relationship between growth curve and product formation.

In the exponential phase, the esterase production was not obtained in significant quantities. Similar reports showed that esterase production by strain LY5 that started from the mid-logarithmic phase reached the maximum during stationary phase [22].

Esterase enzyme was partially purified by common methods namely ammonium sulfate precipitation and dialysis. Comparable work has been done on *Bacillus* cultures procured from culture collection centre by other researchers [5,6,16,21,23,24]. Esterase enzyme was sucessively purified at 80% ammonium sulfate saturation concentration. This was further purified using dialysis tubing method.

SDS-PAGE gel electrophoresis indicated the presence of an esterase having molecular weight of approximately 62 kDa (Figure 3) after silver staining.

Figure 3: Molecular weight of esterase by SDS-PAGE

The moleculr weight of our esterase enzyme was found to be related to the esterase isolated from *Anoxybacillus gonensis* A4 [2]. A wide range of molecular weights (31 to 210 kDa) of microbial esterases is observed owing to the different number of subunits and the types of esterase enzyme.

Enzyme kinetics of the acetyl esterase showed a relatively lower km value which indicates higher affinity of the enzyme towards the substrate. Higher catalytic efficiency is a measure to prove the efficiency of acetyl esterase enzyme to convert of reactant into product. According to the Lineweaver – Burk Plot, the kinetic properties of esterase were: $K_{m\,=}$ 0.91mM, $V_{m\,=}$ 213uM/min/mg, $K_{cat\,=}$ 234/min, Catalytic efficiency = 257.14/mM/min. Variable km values are seen in different esterases isolated from different sources [2,6,23,28].

Enzyme stability with respect to pH and temperature was studied. Enzyme activity is affected by a change in pH. The pH optimum range where stable acetyl esterase activity was obtained is in the pH range 6.0 to 8.0 which is near about the neutral pH. This is similar to the report where the optimum pH of the esterase produced by *Vibrio fischeri* was 7.0[33].But there are certain esterases which work in acidic (esterase from *Monascus* spp. is active at pH = 3.5[17]) and in the alkaline pH (esterase from *Halobacillus* sp. strain LY5 is active at pH = 10.0[22]). The enzymes having their optimal pH in the acidic or alkaline pH prove to be of great importance in many industrial processes where such extreme pH is essential. Sometimes, it is difficult to quantify the acetyl esterase activity above the pH 6.5 or 7.0 on p-NPA and α- naphthyl acetate because of its insolubility. 4-methylumbelliferyl acetate was used, which is more stable as compared to p-NPA and α- naphthyl acetate[34]. The optimum temperature of an enzyme is the temperature at which maximum enzyme activity is obtained. Most enzymes start losing their activity as the temperature increases. The acetyl esterase is stable in the temperature range of 30°C to 50°C. This is in good agreement with earlier reports [3,23,24]. However there are certain thermostable esterases which are very important in industrial processes requiring high temperature[2, 33].

One of the recent studies by Carolina Peña-Montes (2013) indicated that the new catalyst namely the recombinant NStcI immobilized in Accurel MP1000. Studies also highlighted the temperature and pH stability of NStcI esterase through the immobilization process. Only few microbial carboxylesterases have been used in synthesis reactions and they show moderate enantioselectivity. Esterase enzyme shows the chemio- and regioselectivity in addition to the enantioselectivity. Such kind of activities has importance for industrial synthesis reaction[37].

Esterase enzyme has been also produced by a thermophilic bacterium like *Geobacillus sp*. AGP-04 which is isolated from hot spring in Bakreshwar, West Bengal, India.is esterase enzyme is responsible for the degradation of some para-nitrophenyl esters. E studied bacterium *Geobacillus sp.* AGP-04 produces a novel thermostable, organic solvent tolerant extracellular esterase which has high stability at a broad range of pH including alkaline scale. The stimulation of enzyme activity in the presence of DTT and mercaptoethanol, and inhibitory effect of Hg2+ shows that –SH groups in specific sites are required. The enzyme production is inducible when fermentation medium is supplemented with polysaccharides and oily substrate as carbon source [38].

TLC method is one of the sucessful methods that assist visualization of separation of components from a mixture. The amino acids present in the protein can be predicted based on the Rf values and colour of spots as compared to standard. The amino acids which were found to be present in the crude esterase enzyme preparation were histidine, serine, proline, methionine. Figure 4 shows the developed TLC plates.

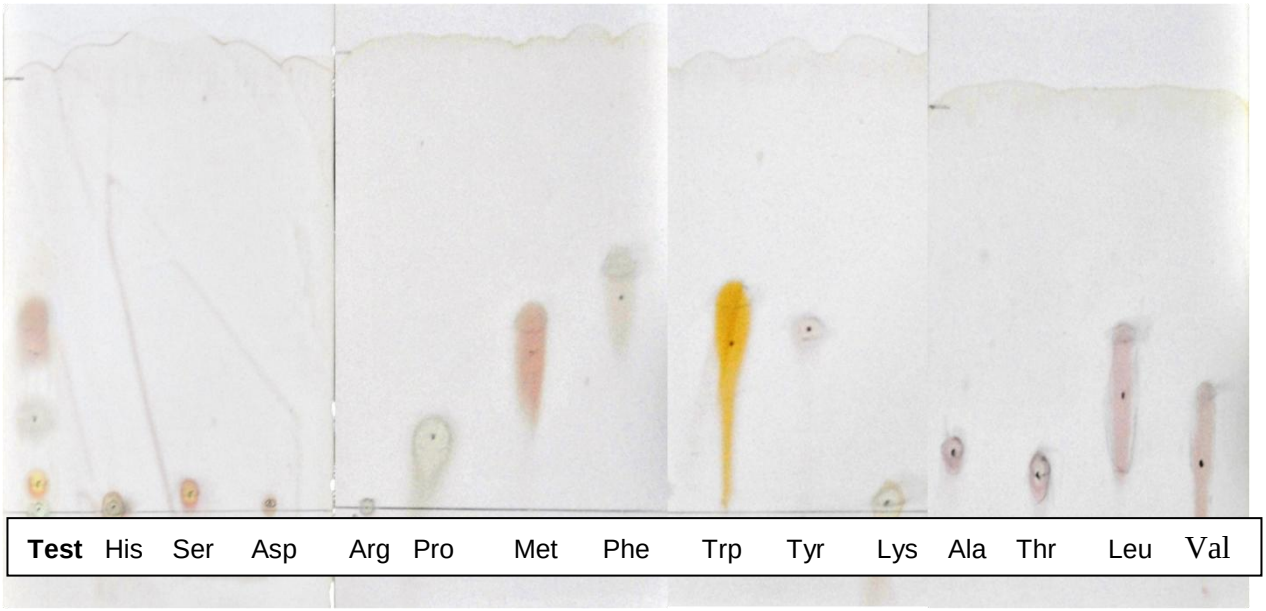

Figure 4: TLC analysis of esterase enzyme showing histidine, serine, proline, methionine in the crude enzyme preparation

The clothing, fabrics industries are always in high demand for synthetic dyes. Most of these dyes are harmful and are not ecofriendly. They are well known as mutagens as well as carcinogens. The toxicity of such dyes can be lowered by various enzymatic, chemical, microbial treatments before their disposal back to the environment. Esterase/lipase enzymes are the most predominant novel enzymes found from the soil metagenome [36,37,38,39]. These enzymes are important biocatalysts for biotechnological applications. One of the remarkable features of lipolytic enzymes includes no requirement for cofactors, and noticeable stability in organic solvents with a broad substrate specificity, stereoselectivity, and positional selectivity [36].

Figure 5 indicates the potential of esterase in decolorization of triphenylmethane dyes. There was no significant decolorization seen in crystal violet

In malachite green, there was visible decolorization. Percent decolorization = 17.18%

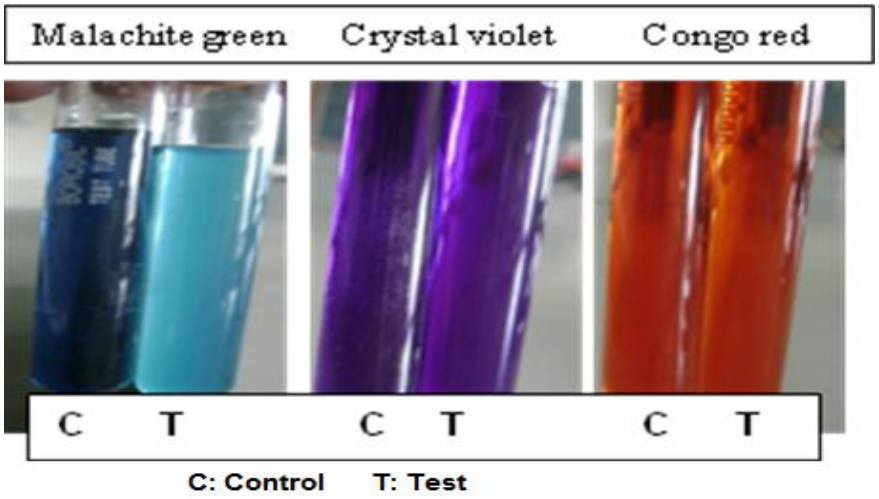

Figure 5: Decolorization of dyes using esterase treatment

A study indicated that the enzymes have higher stability in ionic liquids relative to organic solvents. This is advantageous because the properties of ionic liquids such as negligible vapour pressure, high thermal and chemical stability will be useful in potential industrial applications [38, 40]. In the past few years, there has been an increase in the use of enzymes in industrial processes due to the development of immobilization of whole cells. Certain advantages conferred by whole cell immobilization include reduction in cost required for enzyme purification, greater resistance to environmental changes as well as higher operational stability [2]. Russell et al [40] compared the evolutionary routes by which bacteria and insects have evolved enzymatic processes for the degradation of four different classes of synthetic chemical insecticide. Studies showed selective the advantage of such degradative activities are survival on exposure to the insecticide. In case of bacteria shows selective advantage for access to additional sources of nutrients. However, bacteria have evolved highly efficient enzymes from a wide variety of enzyme families, whereas insects have relied upon generalist esterase-, cytochrome P450- and glutathione-S-transferase-dependent detoxification systems.

Conclusion

In conclusion, *Bacillus* spp. also stands as a representative candidate for production of acetylesterase. Environmental parameter, like temperature of 37°C, pH – 7, ethyl acetate (1%) as substrate concentration produces esterase enzyme of 62 kDa, possessing decolorizing property triphenylmethane dyes namely malachite with a kinetic properties of $K_{m\ =}$ 0.91 mM, $V_{m\ =}$ 213 uM/min/mg ,, $K_{cat\ =}$ 234/min, Catalytic efficiency of 257.14/mM/min. On the other hand, azo dye namely Congo red did not demonstrated any such activity. There is immense contribution to the eco-friendly approaches towards industrial applications due to microbial esterases . In future much of the studies needs to be targeted on esterases. In conclusion enzymatic decolourization of synthetic dyes, esterases also provide a significant decolourization potential.

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