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# REVIEW ON PRODUCTION AND PURIFICATION OF MICROBIAL CUTINASE FOR BIOTECHNOLOGICAL APPLICATIONS

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## ABSTRACT

The present review aims at discussing the structure, function, production of cutinase by using different microorganisms in a fermentation techniques, purification of cutinase and the applications of cutinases.

**Keywords:** Cutinase, hydrolysis, polyester degradation, purification, applications.

## INTRODUCTION

The tender portions of the plants and the epidermis of leaves, shoots and other portions that are found above the ground are covered by the protective coating known to be a plant cuticle. This plant cuticle provides protection to the plants from the infection caused by the pathogens and also from dehydration due the composition of the cuticle, consisting of waxes and polymers, lipid in nature. Lipid polyester that is insoluble in nature is known to be an important component of the plant cuticle and is known as cutin. Fig. 1 shows that cutin consists of 16 and 18 carbon fatty acids that contains hydroxyl group and ester bonds are found to be a source of linkage between them. But this polymer can also be hydrolyzed by the action of enzyme produced by the variety of microorganisms mainly fungi and bacteria and this enzyme is known as cutinases. Previously the action of cutinases was thought to be as an infection to the plants caused by fungi or bacteria. When the spores of fungi come in contact with the cuticle of the plants, it results in the production of the enzyme cutinase due the cutin monomers but in various pathogens the inhibition of these enzymes may also results in the blockage of the infectivity (Kolattukudy *et al*., 1989).

EC of cutinases is *(EC 3.1.1.74).* This enzyme belongs to the superfamily that is alpha/beta hydrolase and esterases of serine. Catalytic triad is found to be present in this enzyme that is serine-histadine-asperginine whereas serine that is catalytic in nature is exposed to solvent. It is an extracellular enzyme that can hydrolyze the polyesters of the cuticle and in addition to its cutinases can also protects the suberin layers, the layers which are involved in the protection of other surfaces of the plants. In between lipases and esterase the enzyme that is thought as an intermediate is cutinases because of the hydrolysis ability of both the enzymes i.e.hydrolysis of soluble esters and lipids. Cutinases are used as a biocatalyst in many reactions like hydrolysis, transesterification and esterification (Carvalho *et al.,* 1999).

Because of the multifunctionality of the cutinases it has a broad range of application like in food, textile, chemical, detoxification, agriculture and many others (Nyyssola, 2015). *Fusarium solani pisi* is considered as a model organism for the complete study and characterization of the enzyme cutinase including its structure, reactivity and function and it was characterized in the 1970s after its identification which was done in 1960s (Longhi and Cambillau, 1999; Egmond and de Vlieg, 2000).

## IDENTIFICATION, STRUCTURE AND FUNCTION OF CUTINASES

### Cutinase identification

For the first time cutinase was found in the fungi that was pathogenic in nature and also from yeast like *Cryptococcus* but later on it was observed and then isolated from *Thermobifida fusca* that is bacteria and it was also isolated from various pathogenic and nonpathogenic fungi (Masaki *et al.,* 2005). Recent work that is done on cutinases isolated from fungal species indicated that these belong to only two subfamilies of the fungi including Basidiomycota and Ascomycota and also from phytophthora that is a water mold but from saccharomycotina not a single species has been found yet (Belbahri *et al*., 2008).

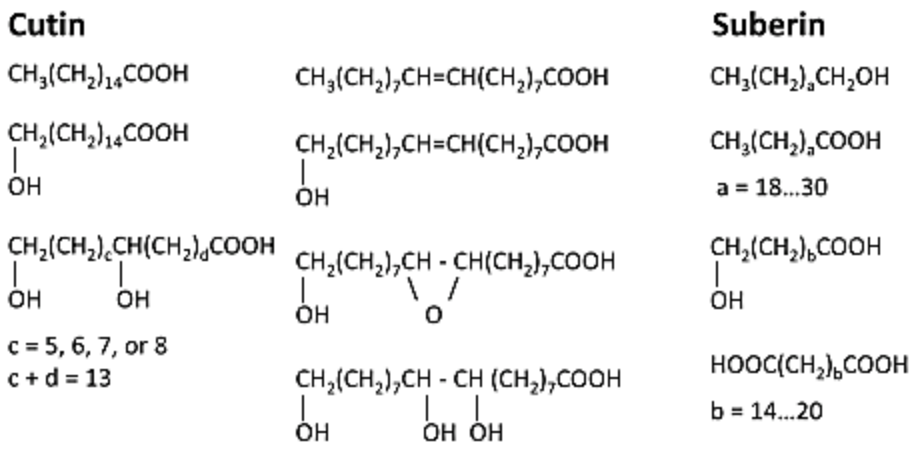


Fig.1. Chemical formulae of cutin and suberin.

**Structure of cutinase**

When solved at the resolution of 1.6 Å and 1.0 Å the three-dimensional structure of *Fusarium solani* cutinase was observed when cloned in *E. coli*with 7.8 of its isoelectric point (Longhi *et al*., 1997). Cutinase is a one domain molecule that is compact in nature and its molecular weight is 22,000 daltons having a great conserved stretch and it contains 197-residue proteins having a size of 45x30x30 Å3. In its conserved regions it contains four cysteins having two bridges that are disulphide in nature (Petersen *et al.,* 1997). Cutinase consist of a beta sheet in the center and either sides of the sheet is covered by five parallel strands which are covered by two and three helices. Gly-Tyr-Ser-Gln-Gly is a stretch that contains ser 120 active site and it has a resilient similarity with the consensus sequence that is known to be from lipase family and it is Gly-(Tyr or His)-Ser-X-Gly. Up to 40 different X ray strutures of cutinases have been solve including their mutants and inhibitor conjugates (Martinez *et al*., 1993; Longhi *et al.,* 1996).

**Function of cutinase**

As discussed earlier that the function of cutinase that is the hydrolysis as it belongs to hydrolases family mainly those that acts on the carboxylic ester bonds. Cutin is an insoluble polymeric compound which protects the arial parts of the plants and its composition includes the hydroxyl along with hydroxy epoxy fatty acids. It is an extracellular degradative enzyme that results in the pathogenicity or infection of the plants as it leads to the degradation of the cutin which then allows the penetration of the fungus and bacteria thus results in infection and release of cutin monomers (Longhi *et al.,* 1996; Jelsch *et al*., 1998).

**PRODUCTION OF CUTINASE**

Cutinase production can be done by variety of techniques by using microorganism of interest that is capable of producing cutinase. The most widely used technique is fermentation.

**Fermentation techniques**

Cutinase production can be done by variety of techniques by using microorganism of interest that is capable of producing cutinase. The most widely used technique is fermentation. SSF is an important technique which is used for the production of cutinase. As SSF is used for the production of microbial origin of many products like medicines, food etc. Microbial enzymes can be produced by solid state fermentation (Kumari *et al.,* 2016). It has many disadvantages including the longer time of the fermentation and less productivity so it is not produced in enough concentration to be used for industrial scale application. Submerged fermentation is another technique that is used for the production of cutinases. It is totally different from solid state fermentation. SmF holds tremendous potential for enzyme production. SmF is favored over SSF for its easier handling, no prior treatment required, better monitoring, feasible control of parameters, and easy purification of products (Hansen *et al*., 2015). And it is appropriate for the growth of those microorganisms during the fermentation process that need greater moisture content.

**Production of cutinase from microorganisms**

Table 1 shows that there are many microbes which produce cutinase and many more are expected to produce this useful enzyme. In prokaryotic bacteria and eukaryotic fungi, a virulence factor is reported to be responsible for the cutinase interdomain gene transfer. Submerged fermentation (SMF) is mostly used for the microbial production of cutinase and solid-state fermentation (SSF) is reported in some cases as well. There some parameters which influence the production of cutinase like temperature, pH, concentration of nitrogen and carbon and dissolved oxygen concentration. In the given Table 1, mentioned below show some microbes responsible for the production of cutinase.

Table 1. Different cutinase producing microorganisms.

| Cutinases | PDB ID | Microorganism | References |
|---|---|---|---|
| Fungal cutinases | 1CUS, 2CZQ, 3DCN, 3GBS, 4OYY, 4PSC, 5AJH, 5X88 | *Fusarium solani pisi, Crytococcus sp., Glomeralla cingulate, Aspergillu soryzae, Humicolain solens, Trichoderm areesei, Fusarium oysporum.* | Nyyssölä (2015); Dimarogona *et al*. (2015). |
| Bacterial cutinases | 3VIS, 4EBO, 4CGI, 4WFI, 5LUI, 5LUJ, 5G0 | *Thermobfida alba, Leaf branch compost bacterial cutinase, Thermobfida fusca, Saccharomono sporaviridis, Thermobfida cellulosilytica, Ideonella sakaiensis.* | Degani *et al*. (2002); Chen *et al*. (2010). |

**Production from fungi**

Cutinase is a cutin degrading enzyme, reported to produce by fungi which are pathogenic in nature. *Aspergillus oryzae* and *Fusarium solani* are frequently used for the production of cutinase. An enhanced thermostability, improved hydrolytic activity, alteration in substrate specificity and degradation of certain synthetic polyester polycaprolactone, are features present in cutinase of *A. oryzae*. The produced cutinase enzyme from *A.oryzae* is represented as*cut L* and it play vital role in formation of flavor. *F. oxysporum* which contain carbon and nitrogen sources is also responsible for the cutinase production.

**Production from Bacteria**

As compare to fungi, very few bacteria are responsible for cutinase production. Two of them are mentioned here that is *Streptomyces scabies* (filamentous plant pathogen) and the other one is strain of plant epiphyte *Pseudomonas mendocina* (Fett *et al.*, 1992).

**PURIFICATION OF CUTINASE**

As cutinase play vital role at industrial scale and have wide range application so its enhancement is mandatory, by keeping its demand rate some of purifying techniques are used to get the partially purified cutinase from the crude one as mentioned in Table 2. At 0-4°C, maximum steps of purification carried out and chromatography of protein done at 280 nm. Following are some methodologies used for purification of cutinase.

**Ammonium sulphate and acetic acid precipitation**

After proper incubation overnight and with constant stirring, a fine powdered form ammonium phosphate is added in 20-80% saturation. At 10,000 g for 30 min, centrifugation was performed and collected the precipitates. Then 20Mm of Tris HCL buffer (pH 8.0), the same buffer is used against dialyzed. In acetic acid precipitation, 30% of it (v/v) is added in small volume and performed on sample which is dialyzed, maintained at pH 4.7. It is one of the most effective processes for purification of cutinase obtained from F. solani (Sebastian *et al*., 1987). Following conditions are maintained in this regard overnight, stirred mixture at 4°C, centrifugation at 14,000 rpm for 40 min. Two fractions are formed on divisin of supernatant. Tris Hydro Chloric acid buffer (20 mM) at pH 8.5 is used against for dialyzation and same buffer 9.5 pH is used for other dialyzation

**Chromatography**

A DEAE (Diethyaminoethyl) Sephacel column is loaded with fraction of pH 8.5 and pre-equilibrated with 20nMTris-HCl buffer at 8.5 pH.NaCl gradient 0-0.1 is used for elution. Tris-HCl 20mM is used as buffer at 7.6 pH, dialyzed overnight against it and was pooled with NPBase activity. The obtained sample put on SP (Sulphopropyl)-Sephadex column, the same buffer of pH 7.6 is used for pre-equilibration. NaCl gradient 0-0.1 is used for elution. NPBase activity is possessed and assayed for activity of TBase. DEAE column is loaded by pH 9.5 fraction. The column is pre-equilibrated 20mMTris-HCl. To 0.1 NaCl gradient is used to check NPBase activity.

**SDS-PAGE**

Sodium Dedocylsulphate polyacrylamide gel electrophoresis analysis is used foridentification of molecular weight of cutinase and its purity. According to Adiguzel and Tuncer, the SDS-PAGE running, preparation of gel and formation of protein bands.

Table 2. Different purification methods.

| Methods used | Recovery % | Fold Purification |
|---|---|---|
| Acetone precipitates | 78 | 3 |
| DEAE-52 | 50 | 8 |
| QAE-Sephade | 9 | 78 |
| Fraction eluted with phosphate | 16.2 | 2.6 |
| Fraction eluted with acetate | 6.5 | 3 |
| Fraction eluted with ligand | 65.6 | 14.2 |

**CUTINASE APPLICATIONS**

Owing to multifunctionality cutinase have potential applications in oil and dairy products, flavor compounds, phenolic compound production, insecticides and pesticides degradation, food industry, in polymer chemistry, textile industry and laundry. Few applications of cutinase are briefly described in Table 3.

Table 3. Applications of cutinase.

| Reaction | Substrates | References |
|---|---|---|
| **Hydrolysis** | | |
| Triglycerides | Triolein<br>Tricaprylin | Goncalves *et al*. (1995) |
| Esters | • ρ-Nitrophenyl butyrate<br>• ρ-Nitrophenylvalerate<br>• ρNitrophenylpalmitate<br>• Methyl-, ethyl-, propyl propionate | Lamare *et al*.( 1997) |
| **Synthesis** | | |
| Esterification | • Oleic acid and hexanol<br>• Butyric acid and 2-butanol<br>• Oleic acid and glycerol<br>• Butyric acid and hexanol<br>• Lauric acid and pentanol | Sebastian *et al*. (1992) |
| Transesterification | • Methyl propionate and propanol<br>• Butylacetate and hexanol<br>• 1-phenylethanol and vinylacetate (racemic miture) | Carvalho *et al*. (1997) |

**Industrial applications**

The industrial applications are further divided into food, agriculture, dairy product, textile, laundry and chemical industries. Being a potent catalyst cutinase used in food industry where it is responsible for the production

of dehydrated fruit, few flavor compounds and some crucial compounds like eicosapentonic acid (EPA) and docosahexaenoic acid (DHA) (Dutta *et al*., 2009). In the field of cardiovascular and autoimmune diseases, these fatty acids (EPA and DHA) shows therapeutic action. In food industry, hydrolytic enzyme is used for the biologic degradation and industrial waste (Gandhi, 1997). Foul smelling residues found in the meat and dairy product industries are responsible for damage intrinsically and extrinsically industrial units can be lesson by using hydrolytic enzyme to degrade the waste. In this waste treatment there is decrease of lipid content hence 60% reduction of effluent is observed in the industrial unit (Leal *et al.*, 2002).

**Detoxification**

The agriculture field required pest control in terms of pesticides and insecticides. For control of plaque and cattle parasite application of organophosphate is required, which are often applied in agriculture field. However, later it was studied that by application of organophosphate has number of adverse effects in invertebrates and vertebrates like mutation is observed in lymphatic cells and blood of humans (Galloway and Handy, 2003).There is need of detoxification and degradation of organophosphate malathion, which is done by using cutinase from *F. oxysporum* and yeast esterase. With the usage of these enzyme within 15 and 30 min of reaction, there is 50 to 60% degradation of malathion (Regardo *et al.*, 2007).

The low water activity property of cutinase can be utilized in oil and dairy product industries, we can acquire transesterification of fats and oils and stereoselective esterification of alcohols (Macedo and Pio, 2005). The highest activity of cutinase against short chain fatty acid was observed. Aflatoxin produce by *Aspergillus*, which can damage crops used as feed. Thirteen (13) different types of aflatoxin are found yet and they contain ester bond. Cutinase act on the ester bond of aflatoxin and degrade it to 51 to 38% at pH 4.5 to 7.0 (Viksoe-Nielsen and Soerensen, 2009). It is important to know the toxicity rate of product in detoxification but unfortunately in the aflatoxin and zearlenone we cannot identify the toxicity. Structural formulas of melathion and aflatoxin are given in Fig. 2.

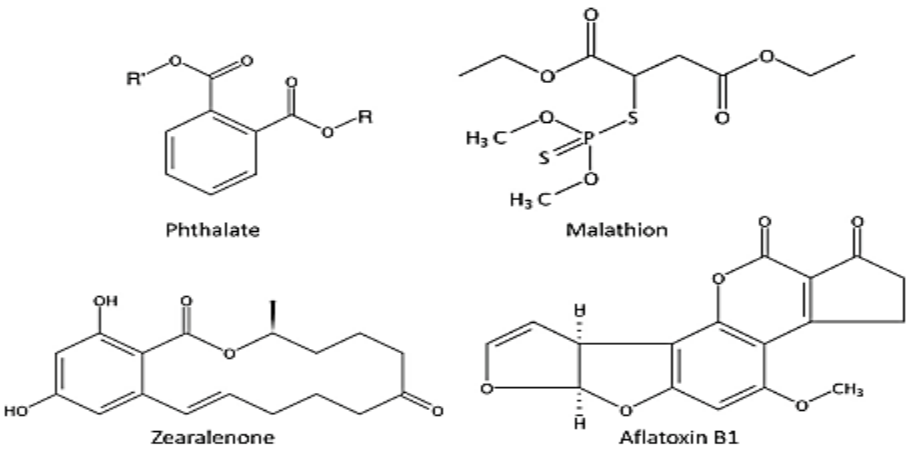


Fig. 2. Structural formulas.

**Textile and laundry industries**

A large part of industry is dependent on cutinase for the ease of work. Like in cotton fiber making industries, cutinase facilitate the uniform dyeing and finishing of cotton by providing wettability. Cotton fiber has complex composition having cutin. Pectin. wax and protein in cuticle layer, which can be hydrolyzed by using cutinase (hydrolytic enzyme) and increase the wettability instead of using alkali (Agrawal *et al.*, 2008; Yang et al., 2011; Zhang *et al*., 2010, 2011). Cutinase degrade cellulose, chitin and both natural and synthetic polymer.

PET (Polyethylene terephthalate), common thermoplastic polymer is hydrolyzed by cutinase, it partially hydrolyzes the ester bonds and release carboxylic group and hydrophilic hydroxyl. Similarly, polyamides are

hydrolyzed or modified by protease and cutinases (Silva *et al*., 2005; Heumann *et al.*, 2009). Wool fiber, wettability effected by using protease and show fiber damage as well. By using cutinase as a pretreatment, give better results in comparison to proteases. Better wettability and shrink resistance with less loss of strength. In laundry and dishwashing, cutinase is used as lipolytic enzyme to remove the stain of fats. In oxidizing condition of the detergent and in the presences of protease, cutinase is generally stable. General mechanism of polymer degradation by cutinase is given in Fig. 3 and Fig. 4.

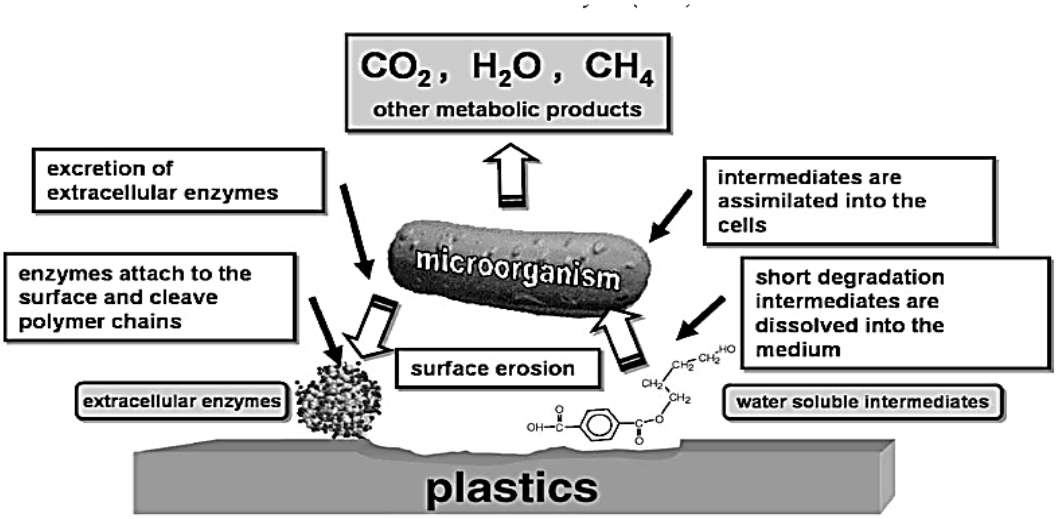


Fig. 3. The general mechanism of enzymatic catalyzed hydrolytic polymer degradation (Mueller, 2006).

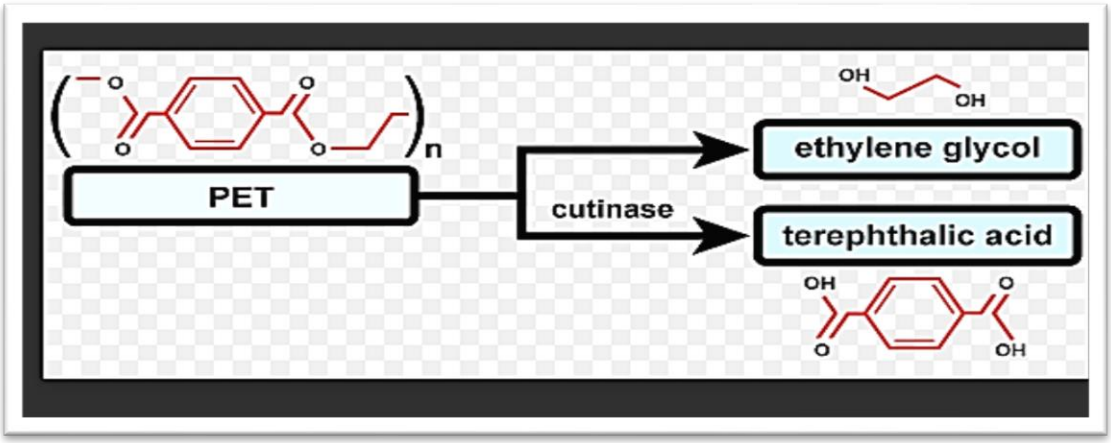


Fig. 4. Cutinase action on PET. (Dutta *et al*., 2009)

**Production of Phenolic compounds**

Phenolic compounds include cinnamic ($C_6$-$C_3$) and benzoic acid ($C_6$-$C_1$) derivatives. Owing to its specific properties and natural occurrence it gets industrial importance. It is found in fruits, aromatic herbs and vegetables, it is used as chelating agent, antioxidant, antiviral, anti-inflammatory, anticarcinogenic and free radical scavengers. They are restricted to use as lipid-based products due to their low solubility rate. Multifunctional amphiphilic molecules are formed as a result of esterification of carboxylic acid with a fatty alcohol, which greatly enhance its hydrophobicity. This phenomenon is called as liphoplization, which can occur chemically or enzymatically. By applying cutinases, lipases and tannase, the enzymatic lipophilization is studied (Stamatis *et al*., 1999; Soares *et al*., 2003) studied by using various enzymes, including *F. solani*cutinase, the esterification of ferulic acid with 1-octanol is performed which are able to catalyze at relatively high rates, the esterification of cinnamic, p-coumaric, and p-hydroxyphenyl propionic acids.

**Biocatalytic Application of Cutinase**

Cutinase is a lipolytic enzyme used for the catalysis of hydrolytic and synthetic reactions. In dairy industries, cutinase is responsible for the hydrolysis of milk fat, in the formation of structure triglycerides and personal-care products. Selective esterification of alcohols and transesterification of fats and oils is accomplished by cutinase. Cutinase show in vivo cutinolyitic activity which is used for the development of pharmaceutical and agriculture chemicals (Genencor, 1988). Cutinase play hydrolytic role as in vitro, towards a broad variety of ester, ester production from soluble synthetic to triglyceride as insoluble long chain. Cutinase is responsible for hydrolysis of fats in the absence of calcium (Ca+) and thus help in dish washing by removal of triacylglycerol.

**Biodiesel Production**

Ethyl or methyl ester fatty acids are included in biodiesel. One of property of cutinase that is transesterification of vegetable oil and fats is used for production of biodiesel. Transesterification is a process in which there is formation of ester and glycerol, when alcohol reacts with fats or oils. Evaluation is performed to check the effect of cutinase as a catalyst, when hexanol reacts with butyl acetate in an organic media during transesterification (Lamare *et al.,* 1997). Emphasis of using cutinase than lipase for the production of biodiesel is owing to its hydrolytic property which emulsified as well as solubilizes triacylglycerol (Pizarro and Park, 2003). Interfacial activation is not required by the enzyme in this regard.

**Application in Process of Deinking**

Deinking is a process, in which there is removal of printed ink from the paper pulp. This process is one of the important parts of recycling, alkali treatment is required for this purpose, and due to this toxic waste is produced at industrial scale. It is alternative of a chemical process, laccases and cellulases are frequently used for this purpose. Hydrolytic property of cutinase is used for deinking of printed paper due to ester bond presence in polyesters, polyvinyl acetate (PVC) and resins. *F. solani, T. fusca* and *H. insolens* have ability of hydrolyzing polyacrylate. Polyacrylates are responsible for decreasing the recycled paper quality by accumulation on paper while recycling process. Better results are obtained by combined action of amylase and cutinase that is 92.6% removal of ink and 92.2% brightness of ISO) (Hong *et al*., 2017).

**CONCLUSION**

With the increasing demand of the enzyme cutinase due to its vital applications in food, detergent and textile industries as well as its polyester hydrolysis ability, it is imperative to produce this enzyme from novel and safer strains. Random mutagenesis can also be induced by chemical mutagens like MMS in order to enhance the enzyme activity. The wild and the potent mutant strains can be examined under electron microscopy, which reveal themorphological differences between the two strains. Moreover, the enzyme was found to be metal ion dependent as various metal ions had positive effect on the enzyme activity. This enzyme effectively degraded 41% of raw polyester under optimized conditions. The study provides a significant contribution towards the enzymatic degradation of polyester polymers.

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