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# STABILITY AND OPTIMAL LIPOLYTIC ACTIVITY OF SONICATED*PRUNUS AVIUM* LIPASEAND ITS APPLICATION ASFAT DEGRADATION AND HYDROLYSIS OF POULTRYWASTEWATER

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## *ABSTRACT*

*Present work is an optimizedlipolytic activity of sonicated defatted PrunusAviumseed lipases. The characterization and the stability of P. aviumlipase were assayed at incremental steps of pH and temperature. Comparison of fat liberation potential of the Lipases treated and untreated poultry wastewater and mutton fat showed the importance of Avium lipase towards fat hydrolysis. A 300Wsonicated waves in 30 minutes made meal clear for enzyme extraction by removing all interferenceof seeds fat,evidencedby the images of scanning electron microscope.10 % olive oil emulsion ; asubstrate,one-hourincubation time and 4000rpm agitation speed werethe optimal variables for extraction of enzyme amongst the 9 runs of experiments suggested byorthogonal L9 array design. Substrate concentration was found the actual contributor to facilitating the lipase for complete hydrolysis. A 43.19%contribution (ANOVA result) of olive oil: substrate was found responsible form aximum lipolytic activity (1.52µU) of Avium lipaseat pH 6 and $40^0$C.Best stability of lipase wasobserved in n-heptane. A release of 6 times more poultry wastewater fat as compared to acid hydrolysis was the successful application of P. aviumafter 180minutes of the incubation period.Degradation of lipids as increase the %age of monounsaturated acid as compared to polyunsaturated acid (GLC analysis) speculating that P.aviumenzyme can be utilized in oil/ fat,detergentand wastewaterindustry due to fat degradation property.*

***KEYWORDS:*** *Hydrolysis, Lipolytic Activity, Orthogonal Array, Prunus Avium, Scanning Electron Microscope, N-Heptane, Sonic Waves*

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## RUNNING TITLE

***LipolyticActivity and the Important Application of PrunusAviumLipase***

## INTRODUCTION

Lipase is the enzyme known as triglycerol hydrolases. The subsequent ability to release the glycerols and fatty acids make lipase participation vital in worldwide enzyme industry market. Moreover, being regioselective, chemoselective or enantioselective, the lipasesare becoming more and more important for special catalytic reactions in bio-technological controlled manufacturing processes [1].Versatility for reactions like hydrolysis, esterification,

interesterification is another reason for lipase acceptabilityin food, detergent, cosmetic, pharmaceutical, leather, lubricant, polyester industries [2].Most recent participation of lipases in the production of bio-modified fats is the unique characteristic of this enzyme which opens the new era of value addition in already existed fatty acids based products to meet the nutritional and safety requirements of the current global dietary rules [3].

Lipasesfate for its suitability and acceptability in different processesisvery much dependent on their sources.Presently, available lipases main sources are gastric, hepatic and pancreatic parts of the animal, bacterial and fungal microorganisms and plants seeds [4]. The selection of the source dependent fully on their catalytic properties towards different reactions like sterification, interesterification, and transesterification includingacidolysis and alcoholysis in industrial application [5, 6].Amongst all plant seed lipasessourcehas been focusing the attention due to its low cost, huge and diversity of sources (plants), easy handling and simple purification methods alongwith the specificity for the targeted reactions [7].Nevertheless, the heat independency characteristic of lipase activity makes it more important for oil chemical industries to cut down their energy expenses and the degradation of compounds as compared to chemical processes. Literatureevidenced the advantages of seed lipases over the animal and microbial lipases for hydrolysis of oils and fats and its application in food, oil chemicals, detergent, and fine chemical industries [3].Recently, increasing interest has been observed of these enzymes in wastewater treatment to reduce sludge flotation after reducing fat and fatty matter concentration which enable smooth aerobic and anaerobic biological treatments [8].The plant source lipase would make the wastewater treatment feasible as compared to high-cost lipases preparation of microbial and animal source.

Oil-bearing seeds worldwide used as the source of oil as well as for the production of biodiesel with the representation of serious industrial waste environmental concern of the defatted seeds (meal). Previous researchers suggested the lipases preparation from different nonconventional plants but no such study was reported on the *prunus* species cultivated tremendously almost all the region of the world. As *Prunus*speciesdemonstrated good fruit nutritive attributes as well as other values: physicochemical, fatty acid profile, antioxidant and antimicrobial so their seeds were chosen for the preparation of lipase enzymes. Ongoing study based on the extraction of enzymes by means of low heat extraction technique –ultrasonic assisted extraction for enzymatic activity(lipolytic) of *Prunus Avium* indigenous to Pakistan. Species under investigation has grown in the northern area of Gilgit and Baltistan. As the total production consumed in the home market, so ample raw material available for the industries for the extraction of the enzymes.Multivariate parameters used to achieve the optimal conditions for ideal lipolytic action with the tolerance power of this enzymes in different solvents.

Characterization of *P. avium* enzyme, its ability to hydrolyze olive oil and theapplication of extracted enzyme as fat degradation of meat and poultry wastewater is the part of the study. The findings will helpto produce a vital industrial product – enzymes with optimal conditions which can be important for making the industrial procedures feasible. The other fruitful outcome will be the utilization of agro-waste which is the main issue of food processing units.

## MATERIALS AND METHODS

**Collection and Defattification of Seeds:** The fruits of *Prunus Avium*were collected from the Karim Abad region of Gilgit-Baltistan- Pakistan. Seeds were collected manually from the fruits grown in garden and orchids. Decortication was done with a 20% salt solution [9].Collected seed kernel was kept in the dry forced oven (Memmert Germany) at $50^0$ C for six hours.Theywere ground into fine powder. Ultrasonic assisted extraction employed for complete defattification of seeds. Hielscher ultrasonic UP 400 S used for sonication. A lipid was extracted by opting conditions after optimization of

different parameters like solvent and seed ratio (S/S ml/mg), sonic power and solvents used. The conditions used for seed defattification for the present study was 300W sonic power, 10ml/gm s/s ratio and a mixture of chloroform and methanol (2:1v/v) were used as the solvent.Extraction time was 30 minutes and the temperature was $50^0$ C[10]. The images of the *Prunusavium* seeds after oil extraction were scanned with Scanning electron microscopic at 25.0kVx1.00kmagnification factor.

**Optimal Extraction of Enzymes by Taguchi Method/Orthogonal Array (Design of Experiment):**Nine experiments were designed by using the advanced statistical technique (L9 orthogonal array of Taguchi method)foroptimal extraction of lipase from *PrunusAvium*. The experimental runs based on three variable parameters with three levels namely substrate concentration (10, 20 and 30 %), incubation time (30, 40 and 60 minutes) and agitation speed (2000,3000 and 4000 rpm) as mentioned in table-1.For each experiment 20gm, defatted seeds of *Prunus Avium* were suspended separately in 0.1M citrate buffer at pH 6.0.The mixtures were shaken in an orbital shaker for an hour at $45^0$ C.The extracts centrifuged with different agitation speed and time based onthe statistical model. The supernatant was diluted further with 1:1 v/v ratio of distilled water and 0.1M citrate buffer. The extracts containing lipases were stored in a refrigerator for enzymes assays [11].

**Enzymes Assays:**The spectrophotometric method was used for the determination of lipase activity. Olive oil emulsion of different concentration (10-30%) was used as the substrate for lipase activity.Assay mixture containing 5ml substrates emulsions, 5ml chloroform, hexane (1:1v/v) mixture, 2.5mlCu–TEA(Copper-Triethanolamine)reagent. Mixtures were centrifuged for 30-60 minutes at 2000-4000 rpm according to experimental conditions (optimization process). An absorbance of supernatants of all the individual experiments was measured at 440nm after adding 0.5ml of 0.15%sodium diethyldithiocarbamate. Stearic acid fractions used as the reference material for enzyme assay.

## LIPOLYTIC ACTIVITY EVALUATION

**Effect of pH and Temperature:**Optimum pH for the lipolytic activities of lipases was determined by using phosphate and trisbuffer covering the range of pH between 3.0-9.0.For optimum temperature absorbance were measured after every 5˚ rise of temperature from 30 to 70˚C while adopting the same method mentioned above in enzyme assay [1].

**Enzyme Stability in the Presence of Different Solvents:**The stability of lipase was studied in the presence of di-isopropyl ether, n-heptane, and cyclohexane by incorporated each solvent into the assay mixture and tested the stability using 10% olive oil emulsion.The pH of the assay was maintained at 6.0 and 40˚C was the temperature [12].

**Hydrolysis of Poultry Waste Water:**Poultry wastewater was collected from a local poultry processing industry (K&N Manga Road, Pakistan). Five samples were treated with different concentrations of enzyme extracts (5-15mg/mL) added to 0.5M citrate buffer for 180minutes at pH 6.0 and 40˚C. The comparison was made with acid hydrolysis of the same waste-water.

**Degradation of Mutton Fat:**Muttonpieces were collected contained fat particles from the local slaughterhouse (Baker mandi, Pakistan).Dissolved the 5gm, piece in enzyme extract (40mg/mL) and incubated at 40˚C for 72 hours. The pH of the mixture was maintained at 6.0. The change in the particle size measured as the decrease of particle mass. The methyl ester of the enzyme-treated as well as control (without treatment) was prepared by using BF3 – methanol as mentioned in the previous work [13].

**Statistical Analysis:** All the experiments were conducted in triplicate. Signal to noise ratio (SNR) was used to

determine the maximum extraction of enzymes from the *PrunusAvium*by considering larger the better (LTB) formula for each experiment (Table2)

Larger the better–$SNR_i$= $-10\log\frac{1}{n}\sum_{j=1}^{n}\frac{1}{Y_j^2}$[1](1) Whereas,analysis of variance (ANOVA) used to determine the %ageparameter contribution towards the optimal extraction of enzymes(Table3) with the help of below mentioned formula

%age contribution of a factor =SSf /SST x100(2)

## RESULTS AND DISCUSSIONS

**Extraction of Oils:**Plants seeds are not only the main source of oils, protein, carbohydrates, and minerals but also produce enzymes utilized by the developing embryo during the storage time in the form of fatty acid [14]. Enzymes activity indicated by the hydrolysis of glycerides and experimentally evidenced by the liberation of the free fatty acids. The concentration of these free fatty acids attributed the lipolyticactivities of lipase in the seeds bearing polar and neutral lipids. The amount of extracted oil by UAE from seeds of Prunus *Avium*were42.75 %.The reason for UAE selection was the previous research work which supported that ultrasonic-assisted extraction (UAE) was a highly efficient method for seeds oil extraction [15]Secondly,a technique was ideal for isolation the seeds compounds present in minute quantities and sensitive to heat [16]. The present study focused on the seeds which itself contain rich amount of oil and free fatty acid so, defatification was done prior to the enzymatic assay. Scanning electron microscope (SEM) images at magnification power 25.0kVx1.00k ensured the complete removal of lipid and free fatty acids of ultrasonically treated *P.avium* seeds (figure-1).

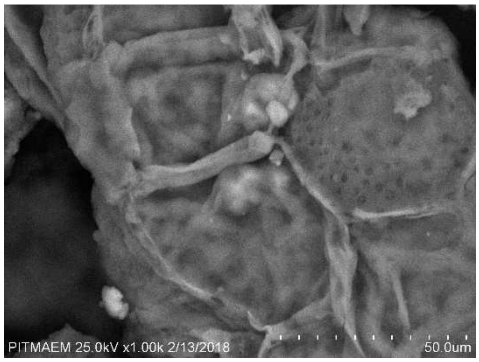



**Figure 1: Scanning Electron Microscopic Image of Sonicated Defatted PrunusAvium Seeds**

**Extraction of Enzymes:**Incubation time, temperature, pH, nature of buffer,agitation speed (rpm) were found the influential parameters of enzymes assays from previously reported research findings. As the amount, conditions and index of pH vary from seeds to seeds so, optimization of the process was essential to achieve the target of thepresent research work.Nine designed experiments were run withincubation time, the concentration of the substrate andagitation speed (variable parameters)along withtheir levels (low to high values). The concentration of free fatty acids showed the action the performance of the enzymes as these enzymes have been recognized as the hydrolysis promoters [17]. A concentration of liberated fatty acid (µL)as a result of hydrolysis of fats of each experiment (in triplicate ).The selection of the Taguchi method for the present study was on the basis of this model version for investigating the production problems and the loss function[18].Loss of function calculated the differences between experimental and predicted values and this was only possible to calculate the signal to noise ratio (SNR).The SNR values were the probable outcome served as the objective of

[1] I,j and n stands for experiment no,trial no and no of experiments

this optimization study. The variable parameters, the concentration of liberated fatty acids, their mean values,SNR and SNRT were mentioned in table-1 by successfully adopting L9 orthogonal array with 9 runs(Taguchi model).

**Table 1: L9 Orthogonal Array Design (Taguchi Method) for Concentration of Free Fatty Acids (µl) andSNRs due to Variable Parameters**

| Experiments | Concentration of Substrate(A) | Incubation Temperature (B) | Agitation Speed (C) | Concentration of Free Fatty Acid(µl) | | | MeanValues of Fatty Acids (µl) | SNR | SNRT |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | | | |
| 1 | 10 | 30 | 2000 | 110 | 108 | 112 | 110 | 40.87 | 44.3 |
| 2 | 10 | 45 | 3000 | 195 | 203 | 202 | 200 | 46.0 | |
| 3 | 10 | 60 | 4000 | 277 | 268 | 265 | 270 | 48.62 | |
| 4 | 20 | 30 | 2000 | 171 | 178 | 191 | 180 | 45.08 | |
| 5 | 20 | 45 | 3000 | 203 | 202 | 200 | 202 | 46.09 | |
| 6 | 20 | 60 | 4000 | 238 | 234 | 231 | 234 | 47.4 | |
| 7 | 30 | 30 | 2000 | 106 | 108 | 110 | 108 | 40.66 | |
| 8 | 30 | 60 | 3000 | 121 | 117 | 123 | 120 | 41.60 | |
| 9 | 30 | 60 | 4000 | 132 | 125 | 138 | 132 | 42.36 | |

To achieve an optimal combination of the variable parameter with an effective level, signal to noise level-basedvalues (SNRL) calculated. SNRL values of each experimental variable for each of its specified level separately mentioned in table-2.

**Table 2: Level Mean SNRs of Variable Parameters for Specific Level**

| Parameter ID | Parameter Description | Levels | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| A | Concentration of substrate | 45.16 | 46.19 | 41.54 |
| B | Incubation time | 42.20 | 44.56 | 46.13 |
| C | Agitation speed | 42.17 | 44.56 | 46.13 |

The SNRL values of each independent variable (A, B, C) at their levels (1, 2, 3) showed the direct influence on the concentration of fatty acids (CoFA). Bigvalue of SNRL greater the influence of that variable of that level to perform the hydrolysis activity of enzymes on the substrate. Analysis of variance (ANOVA)applied to determine the significant contribution towards the maximum CoFAat optimal levels represented in table-3. The values obtained after taking the sum of the square of the fifth factor (SSf) and the total sum of square (SST) as calculated by earlier researchers [18].

**Table 3: Parameters Contribution towards Optimal Cofa**

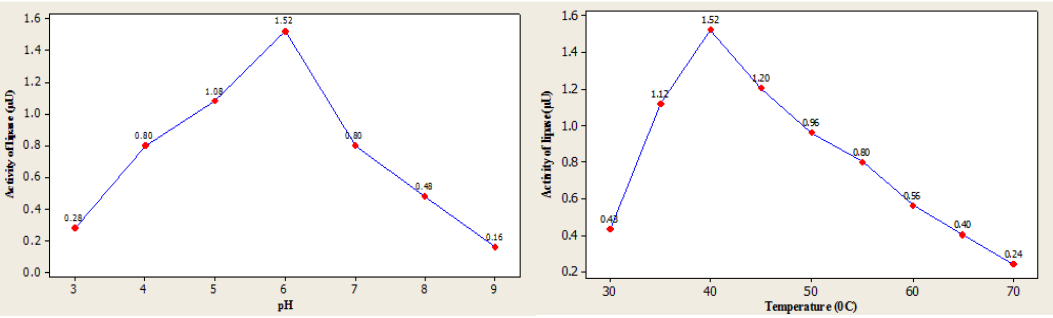
| Variable Parameters | Significant Contribution in terms of Percentage |
|---|---|
| Concentration of substrate | 43.19 |
| Incubation time | 28.16 |
| Agitation speed | 28.65 |

The concentration of the substrate found the more significant parameter in the present study as compared to incubation time and agitation speed. The optimal concentration of the substrateswas found in most of the enzymatic studies [19].

**Lipase Characterization:**Thelipase characterization based on its action and the lipolytic activity is the attributor of lipases activity, soevaluation of the activity was made by monitoring the enzymes activities and stabilities at various temperature, pH, and solvents. Prior to taking the absorbance of liberated fatty acids of *Prunus Avium* a standard curve of different concentrations of the stearic acid plot was drown (50-500 µL). The selection of stearic acid was made due to stability and easily availability according to local climate and environment. Previous work reported the successful use of

stearic acid [20] along with other fatty acids (oleic acid) as the control reagent for enzymatic studies [21]. Literature revealed that characterization of enzymes influenced by the external factors like pH and temperature moreover; the ripening stage, soil nature, extraction procedure,and seed origin also contributor of enzymes properties.These internal and external parameters make the extracted enzymes viable for different industries, to control the rancidity and increase the shelf life of the product [22]. Variation in pH plays the vital role in the efficiency of lipolytic activity of enzymes. The optimization of the pH for lipases is mandatory step to make the enzymes specific and viable for reaction either for catalyzed synthesis or for the formation of products through reactions (hydrolysis, acylation, esterification, and cleaving).

Different experiments were carried out with the variation of pH covering the range of acidic to alkaline 3.0-9.0.Extracted lipase hydrolyzed the substrate triglycerides(10% emulsion fraction of olive oil) measured as the concentration of liberated fatty acids (LFA) in the form of absorbance. lipase showed single peak point when measured absorbance at different pH which clearly indicated the presence of single nature enzyme, the present finding was in accordance with the previous study of lipolytic activities of extracted lipases of peanut, walnut, castor, lipin, pinus and rape seeds which showed single optimal point of pH [22]. Similarly,Variation in the temperature not only influenced the rate of lipolytic activities of lipases but can also be leading to the total denaturing of the enzymes as well. Grosh and coworkers reported the complete loss of enzymatic activities at elevated temperature [23]. The absorbance of fatty acid corresponding to the different temperature in the present study was measured at 440.0nm.The noted absorbance values at different pH and temperature are the indicators of the carboxyl ester bond cleavage of olive oil with the removal of fatty acids and their comparison with standard curve values of stearic acid showed their concentration. Thelipolytic activity of lipases at different temperature and pH profiled in figure-2 (b&a) after incorporating the LFA concentration in the Guven'sequation[24]




(a) (b)

**Figure2: Effect of Ph (A) and Temperature (B) on P.Avium Lipase Activity LipolyticActivity was Calculated by Measuring the Absorbance of Liberated Fatty Acids using 10% Emulsion of Olive Oil**

The result of present work showed the optimal activity of extracted lipase of *PrunusAvium* was observed at $40^0$ C when assayed at pH 6.0, likewise the stability of lipolytic activities regarding pH was around 4.0-7.0forolive oil substrate,whereas at 35-$55^0$C the *P.avium*lipaseexhibited thermal stability which was in accordance to the previous work [25] on different vegetable oil-bearing seeds. The thermal and alkaline stability range of same origin enzymes can be varying due to the change of the substrate, incubation time and agitation speed.

The stability of enzymes is very much dependent on the solvents because the solvents can alter the pH memory, thermal stability and even substrate selectivity of the enzymes. Gupta and coworker reported inhibition of enzymes activity in different concentration of organic solvents [26]. Di-isopropyl ether, n –heptane, and cyclohexane were used to study

thestability of the *PrunusAvium*extracted enzymes on already observed optimal points of temperature and pH.All the solvents showed stability against these optimal points with a variation of their activities in term of the concentration of fatty acid liberation and their absorbance.The figure-5 depictedthe stability trend of extracted lipases due to the combination of hydrophilic solvent together with a non-aqueous solvent which is in accordance with the Tsuzuki and Kitamura studies [27]

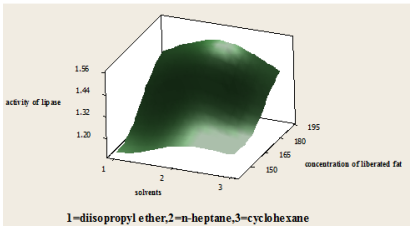



**Figure3: Surface plot of P.Avium Lipase Activity in Different Solvents at $40^0$ C and 6.0 Ph**

**Hydrolysis of Poultry Waste Water:**The wastewater of poultry industries contained organic and fatty based suspended solid fractions. The sources might be the washing steps of slaughter birds, equipment and processing plant washing [28]. The free fatty acids concentration in poultry field waste-water was analyzed before and after the lipolytic activity with different concentration of lipase extract. The maximum concentration of fatty acids was observed with 40mM lipase extract after 180 minutes of incubation time which was six times higher than the control wastewater sample (figure 4).

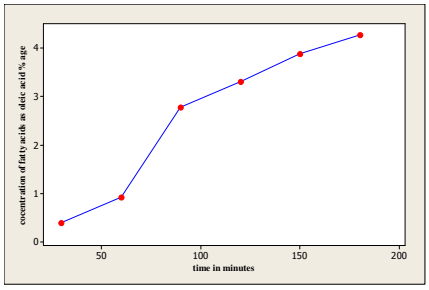



**Figure 4: Effect of Lipase on the Liberation of Fatty Acid of Poultry Wastewater at Different Incubation Times**

Polizellireported the liberation of fatty acids 7.4 times higher with enzymatic treatment than the normal acid hydrolysis [5]. The variation in the activity might be due to the variation of wastewater characteristics of different processing plants, consumption of water for each slaughtered bird and the source of enzymes. The results are important for the poultry industrial wastewater processfor the conversion of triacylglycerols into fatty acids and will step forward for industry development as few studies reported to use lipase in fat wastewater.

**Fat Degradation:**Degradation of the fat particle in flash of animals was reported in different studies with a different concentration of enzymes and at different incubation time, temperature and pH. Using 25mL,*P. avium* enzyme for 5gm, mutton piece, 6 % mass reduction was observed by calculating the weight of the particle. The result did not match with the findings of Masses studies of 40 % reduction of beef particle size with pancreatic lipase and others with no claim of particle size change[29]. The apparent reason of drastic variations of results in case of a mass reduction up to 40% is the source change;pancreatic lipase(animal source of enzyme) which has higher activity as compared to plant source lipase.The fatty acid profile of the lipase treated and untreated mutton revealed the reason for mass reduction. Change in the composition of higher molecular weight fatty acids (linoleic and linolenic acid ) might be the reason for loss of particle

size. Previous work mentioned the activity of lipase on oleic acid as well as linoleic and linolenic acids [30].The change in the concentration of oleic acids and other fatty acids of lipase treated mutton fat and control of the present study was mentioned in table-4.

**Table 4: Fatty Acid Composition of Lipase Treated and Un-Treated MuttonFat**

| Fatty Acids | Fat Particle %Age (Untreated) | Fat Particle % (Lipase Treated) |
|---|---|---|
| Myristic acid | 4.1 | 5.0 |
| Palmitic acid | 18.9 | 20.7 |
| Palmitoleic acid | 1.6 | 0.7 |
| Stearic acid | 30.8 | 31.3 |
| Oleic acid | 39.05 | 39.87 |
| Linoleic acid | 3.57 | 1.7 |
| Linolenic acid | 1.1 | 0.3 |
| TSFAs | 53.8 | 57.0 |
| TUFAs | 45.32 | 42.57 |
| PUFAs | 4.67 | 2.0 |
| MUFAs | 40.65 | 40.57 |

All the results of studied parameters showed the extracted lipase effective activity at optimal pH, temperature and its successful application in fat degradation and wastewater hydrolysis. The difference in the ranges of the lipolytic activity can be due tosome disadvantages likeselection of the substrate for application as shown lipase extraction,an interaction of the existed fat of plant seed,origin of lipase, hydrolysis time and the concentration of lipase.

## CONCLUSIONS

In this study, the organic solvent stable lipases fromPrunusAvium were successfully studied regarding optimal activity at different temperature and pH, along with the specificity and concentration of the substrates. Taguchi method made possible to extract the enzymes after optimizing the parameters like substrate concentration, incubation time and agitation speed. The single neutral nature enzymes with single optimal pH and temperature generated the possibility of the usefulness of this enzyme in fat particle degradation and poultry wastewater treatment.

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