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Research Article

MUTATIONAL STUDY OF *BACILLUS* SPECIES FOR PRODUCTION, PURIFICATION AND CHARACTERISATION OF LIPASE

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ABSTRACT

Lipase being a hydrolyse enzyme acts on lipids to give fatty acids and glycerol. Soil microbes were isolated and screened for the potential producers of lipase using Tributyrine agar plate method. Bacillus lichiniformis MTCC2617 is optimised for comparative study with wild microorganism. Wild and MTCC2617 are inoculated in the production medium contained standardised carbon and nitrogen source. Wild was UV mutated for the strain improvement. The activity of the wild was increased from 780 U/ml to 2120 U/ml for 20mintues incubation. The crude enzyme from wild, mutant wild and MTCC2617 was partially purified by ammonium sulphate precipitation, dialysis and ion exchange chromatography. After the complete purification percentage of yield was high for wild mutated as compare to MTCC2617. The molecular weight of the protein was found to be 55KDa for both MTCC2617 and mutant wild. Characterisation of the lipase enzymes showed optimum pH 9.0 for wild, mutant wild and 7.0 for MTCC2617. Optimum temperature was 55^0C. The enzyme was activated by 9µM of Calcium chloride and in the presence of 2.5µM Ethylenediamine tetra acetic acid the activity was reduced to 12.5% for mutated and MTCC 2617.

Keywords: Ammonium sulphate precipitation, Dialysis, Tributyrine agar plate method.

INTRODUCTION

Triacyl glycerol acyl hydrolase commonly known as lipase, whose natural catalytic function is to hydrolyse ester bonds in triglycerdies to produce diglycerides, monoglyceridies, glycerol and fatty acids. Lipases are ubiquitous enzymes produced by all biological systems via, animals, plants and micro-organisms[1]. They occur widely in nature but microbial lipases are commercially significant[2]. Micro-organisms produce a wide spectrum of lipases that differ in their enzymatic characteristics, such as substrate specificity, pH, temperature activity and stability profile[3]. After proteases and amylases, lipases are considered the third greatest group in sales volume, moving billions of dollars, showing their versatility of application, which makes them especially attractive for industrial applications[4]. This has increased the demand of the wild strain with the maximum production of enzymes.

In the present investigation is to enhance the production of lipases by subjecting the wild low yielding strain to random mutagenesis by UV radiation.

MATERIALS AND METHODS

Isolation of lipase producing organism from soil source

1g of soil sample was serially diluted and the samples were inoculated into the Tributyrin agar containing glucose 2; yeast extract 5; tributyrin 5; sodium chloride 0.4; calcium chloride 2; magnesium chloride 1 (g/L) and incubated at 37^{0}C for 24 to 48hrs. The colony producing the highest clear zone of tributyrine (CZT) was selected. The wild organism was maintained on nutrient agar slants. It was identified by morphological and biochemical characteristics[5].

Standardisation of carbon and nitrogen source in the media

Both the wild and MTCC 2617 was inoculated into different production medium containing glucose 10; maltose 10; glycerol 10 (g/L) as carbon source. The pH of all the production media was adjusted between 7 and 8 and incubated for 24hrs, 48hrs and 72hrs. As above two parameters (temperature, carbon source) kept constant and inoculated with two organic nitrogen sources (soya powder 1% and casein 1%) and two inorganic nitrogen sources (ammonium chloride 1% and ammonium nitrate 1%).

Enzyme assay

The reaction mixture containing 3ml of olive oil emulsion (25ml of 0.2M Tris buffer mixed with olive oil in the presence of an emulsifier) 2.5ml of Tris buffer and 1ml of enzyme solution. The control containing boiled inactivated enzyme (at 100^{o}C for 5min) was treated similarly. After the incubation the enzyme activity was blocked by 3ml of 95% ethanol and liberated free fatty acid was titrated against 0.05M Sodium hydroxide using phenolphthalein as indicator. One unit will hydrolyze 1.0 micro equivalent of fatty acid from a triglyceride in one hour at pH 7.7 at 37°C[4, 6].

Estimation of protein

The amount of protein was estimated by the method of Lowry *et al.*,[7] using bovine serum albumin as the standard.

Purification of lipase

The production media was centrifuged at 10,000rpm at 4^{0}C and the cell free supernatant was the crude enzyme. The lipolytic efficiencies of the crude enzyme were analyzed. All the purification procedures were carried out at 4^{0}C. The crude enzyme was subjected to 70% saturated ammonium sulphate precipitation. The precipitate was collected by centrifugation at 9,000rpm for 15min and dissolved in minimal amount of 0.1M Tris Hydrochloric acid buffer of pH7.0 and then dialysed extensively against the same buffer[8]. The enzyme activities in the dialysate were assayed. The dialyzed fraction was applied to a Diethylaminoethyl cellulose (DEAE-C) column, an anion exchanger, pre-equilibrated with Tris-Hydrochloric acid buffer, pH 8.6. The enzyme was eluted (1 ml/min) with sodium chloride gradient (0.1 - 0.5 M) and 0.025M Tris buffer, pH 7.0[9].

Strain improvement by UV mutation

The bacterial strains were subjected to UV radiation to see its effect on enzyme activity. The strains were cultured in Leuria Bertani broth and 1ml of grown culture was subjected to UV (254 nm) irradiation for 5, 10, 15, and 20 minutes. The exposure was carried out at distance of 30.0 cm away from the centre of the UV light source. Immediately after exposure, the cultures were inoculated into the production medium and incubated for 48hrs. Wild strain from unexposed plates were inoculated into production medium and served as control[10]. After incubation, lipase assays were performed by using the similar procedure

Characterisation

The optimum activity of the enzyme was determined by assaying at different temperatures 4, 28, 37, 55 and 100^{0}C and pH 3, 5, 7, 9, 11. The activity was determined in different concentration of the metal ion Calcium chloride ($CaCl_2$) and metal ion chelator Ethylenediamine tetra acetic acid (EDTA) in the concentration of 1 to 9µM and 0.5 to 3µM respectively in the reaction mixture.

SDS PAGE

SDS PAGE was performed according to the method of[11], with a separating acrylamide gel of 12.5% and stacking gel of 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250 and de-stained with a solution of methanol, acetic acid and water in the ratio of 4:1:5[9]. The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers phosphorylase b (97.4KDa), Bovine serum albumin (66KDa), Ovalbumin (43KDa), carbonic anhydrase (29KDa), lactoglobulin (18.4KDa) and lysozyme (14.3KDa).

RESULTS AND DISCUSSION

Screening For lipase production

In the present research lipase producing organisms were isolated and screened using tributyrin agar plates from oil contaminated soil. Large clear zone colony was selected after 24 to 48hrs of incubation. Lipolytic organisms were screened by qualitative plate assay. The clear zone was because the tributyrin was hydrolysed to form free fatty acids and glycerol[5].

Biochemical investigations for taxonomic identification of bacteria producing lipase

Taxonomic characterisation of the isolated bacterial strain was carried out by applying series of standard Biochemical tests. It has been observed that bacterial sample has shown positive results for Citrate utilization test, Gelatin test, Urease test, Glucose, lactose, sucrose fermentation test, starch, lipid hydrolysis test and oxidase activity test. All the below biochemical tests confirmed that our wild organism may be Bacillus **Table 1.**

Standardisation of carbon and nitrogen source for the optimum production of lipase

The maximum production of enzyme was at 48hrs. After 48hrs the growth showed divergence from the exponential because in place of homogenous growth bacterial pellets began to form in which nutrients and oxygen supply because the growth limiting. This results in the reduction of lipase yield due to the consumption of nutrient materials[12]. As shown in the **Table 2** wild mutant and MTCC2617 production media with glycerol in combination with ammonium nitrate gave the optimum production of lipase is 2270U/ml. In the present research lipase from bacillus species was found to be inducible and its yield was significantly affected by the type of carbon source used. According to Kim *et al.,* 1994[13] glycerol shows maximum lipase activity at pH 7.2 and temperature 55^{o}C. However Gupta *et al*., 2004[14] reported the inducible effect of glycerol on lipase production by Bacillus species

The combination of olive oil, ammonium nitrate and glycerol showed the maximum enzyme activity in 48hrs of incubation. The studies of Anurag Sekhon *et al.,* 2006[3] stated that among inorganic nitrogen sources the best growth and enzyme activity was observed in the presence of NH_4NO_3 (cell density 600nm, 0.635; enzyme activity 110U/ml) in *Bacillus megaterium*[15].

Strain improvement by UV Radiation

The wild organism was subjected to UV mutation where the 20min incubation showed the positive mutation. The activity of the wild was increased from 780U/ml to 2120U/ml as shown in the **fig 1 and Table 3.** UV rays are important inducers of strain mutations. The pyrimidines (thymine and cytosine) are especially sensitive to modifications by UV rays absorption. This may result in the production of thymine dimers that distort the DNA helix and block future replications. In many cases, mutations by UV are harmful, but occasionally it may lead to a better adapted organism to its environment with improved bio catalytic performance. The potential of a microorganism to mutate is an important property conferred by DNA, since it creates new variations in the gene pool[16].

Purification of crude enzyme from mutated wild and MTCC2617

After the complete purification that is after Ion exchange chromatography it was observed that yield was high for Mutated wild (71%) as compare to MTCC2617 (67%), this result shows increase in the lipase production for wild **Table 4.**

SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis)

The isolated enzyme samples (pure sample and crude sample) from wild, MTCC2617, and mutated wild. The molecular weight of the enzyme is found to be 55KDa. Approximately by using marker in SDS-PAGE electrophoresis.

Characterization of enzyme

Effect of pH on enzyme activity

The optimum pH of the enzyme was found to be 9.0 for wild mutated. For MTCC 2617 the optimum pH range was between pH5 and pH9 **Fig 3**. In MTCC2617 at pH 3 and 11 it retained nearly 80% of activity. But in wild mutant at pH 11 the activity was reduced to 8.3%[3], where *B.Megaterium AKG-1* could grow in wide pH range of 4 and 11. However the best yield was observed in pH range 6.5-8.0[16].

Effect of temperature on enzyme activity

The optimum temperature of the enzyme from the different sources was found to be 55^{0}C **Fig 4**.This is in agreement with the results obatained by R. Bayoumi *et al.*, 2007[17] lic B-42 where lipase produced by *B. licheniformis B-42* reached its maximal activity at 50-60ºC where it reached up to 50.23 U/ml. Below or above this range the activity decreased.

Effect of activator on enzyme activity

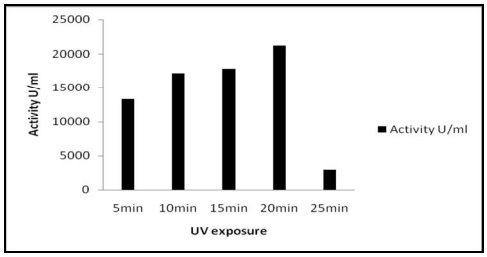
In the presence of 9µM $CaCl_2$ the enzyme activity was increased **Fig 5**.

Table 1: Biochemical tests

Biochemical Tests	Wild
Indole production Test	-
Methyl Red Test	-
Voges Proskuer Test	-
Citrate Utilization Test	+
Urease Test	+
Catalase Test	-
Gelatin Test	+
Glucose Fermentation	+
Lactose Fermentation	+
Sucrose Fermentation	+
H_2S Production Test	-
Starch Hydrolysis Test	+
Lipid Hydrolysis Test	+
Oxidase Activity Test	+

Table 2: Media Optimisation for the Maximum Production of Lipase

	Lipase assay on second day in U/ml	
Organisms	1% Glycerol	1%Glycerol with 1% Ammonium nitrate
Wild	760	780
MTCC2617	1680	2270



Fig, 1: Enzyme activity in different incubation period

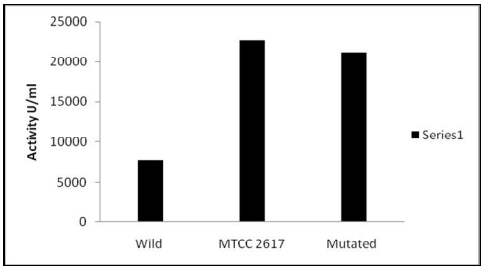


Fig. 2: Strain improvement by UV Radiation

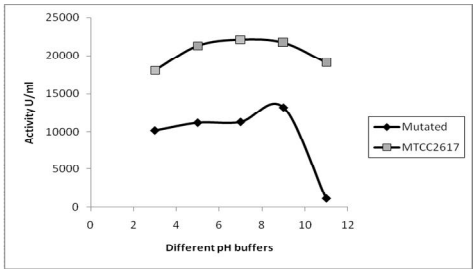


Fig. 3: Effect of pH on enzyme activity

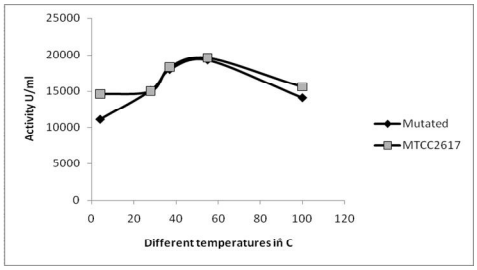


Fig. 4: Effect of temperature on enzyme activity

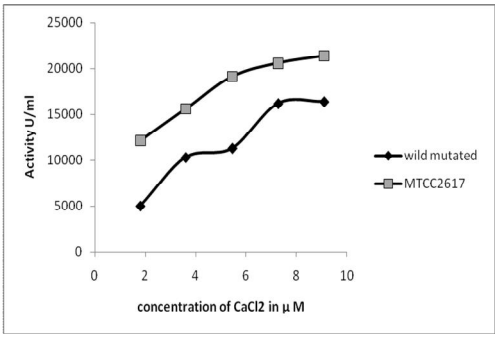


Fig. 5: Effect of activator on enzyme activity

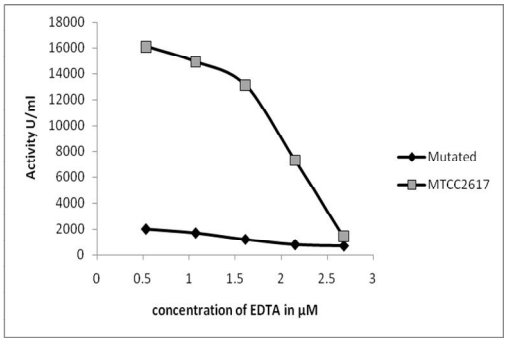


Fig. 6: Effect of inhibitor on enzyme activity

Effect of inhibitor on enzyme activity

In the presence of 2.5µM EDTA the activity was reduced to 12.5% for mutated and MTCC 2617 **Fig 6**. This is in agreement with the work of sangeeta *et al*., (2010)[18] where it showed only 48% activity in the presence of EDTA. Sailas Benjamin, (2000)[19] also reported 75% inhibitory activity in the presence of EDTA. Action of this compound could be attributed to their effect in creating the imbalance of ions in the reaction mixture by absorption or release.

CONCLUSION

It is well known that lipases are the most widely used enzymes in organic synthesis and more than 20% bio-transformations are performed with lipases. In addition to their role in synthetic organic chemistry. There is also extensive application in chemical, pharmaceutical, food, leather industries and biodegradation of plastics. In view of variety of applications, in this study an attempt has been made to isolated wild bacterial sample and to improve lipase production by mutation using UV radiation.

Table 3: Strain improvement study by UV radiation

Mutagen	Protein Conc. (mg/ml)	Enzyme activity U/ml	Specific enzyme Activity U/mg
UV radiation			
5	0.697	1330	$19.08*10^3$
10	0.747	1710	$22.89*10^3$
15	0.752	1770	$23.53*10^3$
20	0.772	2120	$27.46*10^3$
25	0.543	1110	$20.44*10^3$

Table 4: Percentage yield of Bacillus lichiniformis MTCC 2617 and wild mutant

Purification	Organisms	Concentration of protein mg/ml	Activity U/ml	Specific Activity U/mg	Yield %	Fold purification
	Mutant Wild	0.986	2120	$21.50*10^3$	100	1
Crude extract	MTCC2617	0.909	2270	$24.97*10^3$	100	1
	Mutant Wild	0.097	660	$71.13*10^3$	78	7.9
After Dialysis	MTCC2617	0.118	1010	$85.59*10^3$	44	3.4
After Ion Exchange	Mutant Wild	0.043	1510	$351.16*10^3$	71	16.3
Chromatography	MTCC2617	0.131	1530	$116.7*10^3$	67	4.6

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