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RESEARCH ARTICLE OPEN ACCESS

Screening, Isolation and Identification of Thermophilic Esterase Enzyme Isolated from *Rhodococcus SP*: LKE-021

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Abstract

***Rhodococcus sp*. LKE-021 from soil samples of the region of Gangotri (10,000 feet of average height) of Uttarakhand Himalayas, India, produced a thermophilic esterase. The physiological and morphological characteristics of the isolated *Rhodococcus sp*. LKE-021 detected as Gram Positive, rod shape, catalase positive, indole negative, positive to glucose and xylose fermentation test, and can grow on the Nutrient Broth medium. Esterase production confirmed on the basis of spectrophotometric enzyme assay. Taxonomic characteristics *Rhodococcus* confirmed by 16s rRNA gene sequencing.**

Keywords: Esterase, hydrolytic enzymes, *Rhodococcus,* LKE -021, extremophiles enzyme, thermophilic enzyme.

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INTRODUCTION

Esterase is a hydrolytic enzyme found in multiple forms occurring with broad substrate specificity. The hydrolysis of organic esters catalyzing by Esterase. Esterase are surviving in the environment due to their capability. They are extensively distributed in animals, microorganisms and plants. Numerous of them exhibits atolerance against varied substrate which led to the postulation that they have developed to allow access to sources of carbon or to be incorporated in catabolic pathways. Furthermore, esterases also exhibit extraordinary stereospecificity, therefore in fine-chemicals synthesis this enzyme used asstriking enzymes for the synthesis of optically active pure compounds[1-6]. Esterase have another tremendous properties i.e. active and stable in organic solvents which make the unique as there is no need of cofactors[7]. Esterase are broadly used in the food, cosmetic, perfume, chemical, pharma and agricultural industries, by reason of exclusive characteristics like resistant to organic solvents, stereo and substrate specificity[8-10]. Though, nearby some obstructions still to be there for the large scale utilization of esterases in industries, for instance low yield of production, thermal stability, limited pH, and deprived performance in organic solvents[11,12]. There is still need of enhanced their catalytic efficiency on the basis of rational protein design through functional modification of the enzymes in addition to substrate specificity, thermostability and enantioselectivityas required by industries[13-16]. Better understanding of structure and functionalities will also ease rational improvement in purification, isolation and source of microbes. Development of new microbial esterase enzyme is very important in Industrial Application. Esterase are the used extensively in food, pharmaceutical, food and chemical fields. While the thermostable amylaseare widely used in starch Industry in various stages of developments[17]. Industrial and scientific significance of these extremophilic enzyme in recent years has increased excessively due to structural and functional stability of their proteins[12].

EXPERIMENTAL METHODOLOGY

Microorganism: Identification and culture conditions

Pure bacterial culture isolated from soil sample collected from Gangotri region (10,000 feet of average height) of Himalaya, Uttarakhand (India). Before inoculation in sterile distilled water (5% w/v) collected soilsample dried for 72 hat 80°C. Serial dilution performed and aqueous phase was collected and inoculation carried out into nutrient broth (NB). The composition of NB (pH 7.0, gL^{-1}): peptone - 5.0; NaCl - 5.0; yeast extract- 3.0; glucose - 10.0. The Plates were incubated for 5 daysat 60°C. Randomly colonies were selected on tributyrin agar plate and screened for esterolytic activity in solidified NB containing 1.5% tributyrin for 3 days at 60°C. After 4 days of cultivation at 40°C a bright clear zone on tributyrin agar plate achieved[18]. Master culture preserved in glycerol (40% v/v) at -80°C for future use. Identification of bacteria performed by 16s rDNA method of sequencing. Purification and Extraction of DNA were performed by Sigma kit (GenElute™ bacterial genomic DNA), as per the instructions given by manufacturer. Primers 5´-CAGGCCTAACACA-TGCAAGTC as forward primerand 5´-GGGCGGWG-TGTACAAGGC as reverse primer used for PCR amplification experiments. Amplified DNA was purified by Sigma DNA kit (GenElute™ bacterial genomic). sequences available in NCBI database used for 16S rDNA sequencingby using clustalW, and TREEVIEW program (3.0) used for depict phylogenetic position of the isolate. Isolated strain was identified as *Rhodococcus sp*. LKE-021 by partial 16S rDNA gene sequencing.

Quantification and production of Enzyme

Rhodococcus sp. LKE-021 was inoculated in shake flasks method containing modified NB (pH 7.0) containing (g/l): peptone, 5.0; NaCl, 5.0; yeast extract, 3.0; glucose, 10.0, with constant shaking at 135 rpm at 60°C with. After 24 hrs, culture was centrifuged at $10'10^3$r.c.f. for 5 min and extracellular enzyme was recoverin liquid fraction. Activity of esterase enzyme estimated spectrophotometrically by with*p*-nitrophenyl acetate (pNP acetate) as substrate. Enzyme containing 250µL supernatant of culturewas mixed with 200µL of 50 mM McIlvaine buffer (pH 7.2) and 50µLpNP acetate (2 mM) and incubated for 30 min at 60°C. Reaction terminated by adding 500µL of chilled buffer and reaction was immediately terminated. Centrifugation was carried out by centrifuging at $10' 10^3$r.c.f. for 5 min

and suspended particles were extracted. Amount of released p-nitrophenyl acetate (pNP acetate) measured by hydrolysiscatalyzed by esterase calculated at λ^{410} nm compared toblank[19]. Total protein was determine by Lowry's method with standard bovine serum albumin (BSA). Protein concentration determine by taking absorbance at λ^{280} nm during chromatographic purification [20].

RESULTS

Collected soil sample tested for its physical–chemical properties and it have 8.5 pH, temperature 6°C, and moisture content 25%, w/v.

Selection ofEsterasesproducing bacteria

35 soil bacterial pure cultures were isolated from the surroundings of Gangotri region of Uttarakhand Himalaya, India. These isolates showed potential for esterolytic activity and amongst them most potent,one isolate used for further work.

Primary Screening thermophilic enzyme producing thermophile microorganisms based on qualitative test

Isolate screened of novel thermophilicactivity on agar plate containing 1.5% of tributyrin in NB (composition in g/L; beef extract 3; peptone 5; NaCl 5; glucose 10; agar agar 20; pH 7.2-7.4) incubatedat 60°C for three days. Further incubation at 4°C for 4 days was done to bright clear zone on the agar plate (Fig. 1).

Biochemical characterization of *Rhodococcus sp*. LKE-021

In order to identify the microorganisms various microbiological and biochemical tests were performed and their results are summarized in a tabular manner (Table 1).

The physiological and morphological features of the strain *Rhodococcus sp*. LKE-021 was detected as Gram positive (Fig. 2), rod, positive to catalase, negative to indole test, positive against fermentation test for glucose and xylose, which can grow in the Nutrient Broth medium (OD at 600 = 0.679 [1:20 dilution], after 22 h). Taxonomic characterization was describe based on the 16S rRNA gene nucleotide sequences.

Table 1. Results of various tests performed to identify the microorganisms

Tests name		Results
Gram-staining		Positive
Endospore staining		Positive
Shape		Rod
Motility		Motile
Anaerobic growth	Glucose	Positive
	Xylose	Negative
Carbohydrate Fermentation	Glucose	Positive
	Sucrose	Positive
	Lactose	Negative
	Mannitol	Positive
Indole production test		Positive
Methyl red test		Positive
Citrate utilization test		Negative
Voges Proskauer test		Positive
Catalase test		Positive
Urease test		-ve
TSI agar test for H_2S production	Slant	Alkaline
	Butt	Acidic
Casein hydrolysis test		-ve

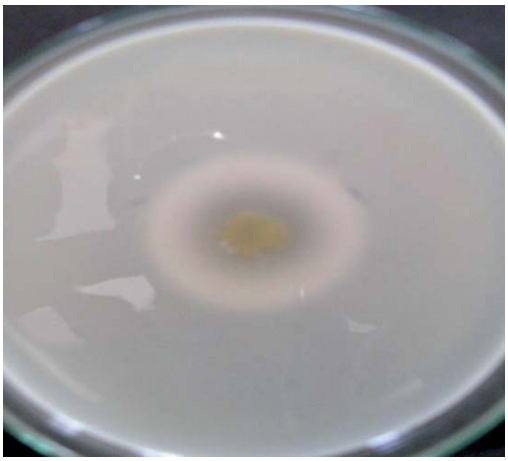

Fig. 1. Primary screening of thermophilic enzyme producing bacteria

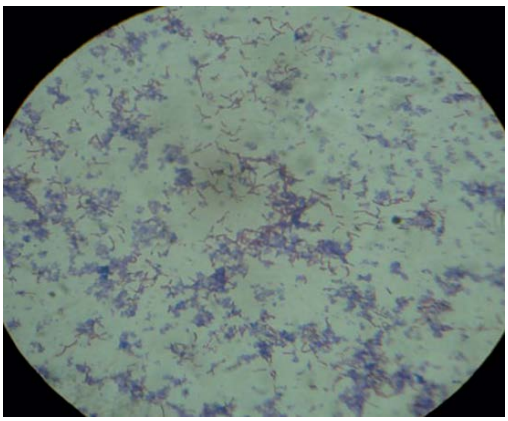

Fig. 2. Gram straining of *Rhodococcus sp*. LKE-021

Optimization of enzyme production

Esterase production was optimized with nutrient broth. 100 ml nutrient broth inoculated with *Rhodococcus sp*. LKE-021 and incubated in 60°C at 120 rpm. Samples were collected from 0 h to 60 h after every 2 h of interval and all the parameters like enzyme production, total protein, growth profile, carbon utilization, estimated of total biomass production in order to optimize standard enzyme production time and condition in shake flask determined. It was observed that maximum activity was attained at around 22 h along with maximum protein production (Fig. 3 & 4). The *Rhodococcus sp*. LKE-021 was at early log phase and total biomass was almost stable. More than 80% sugar (sole carbon source) has been utilized by that time (Fig. 5). *Rhodococcus sp*. LKE-021 was incubated in modified nutrient broth for 48 hour after inoculation at 37°C in incubator shaker at 120 r.p.m. for further laboratory scale purification.

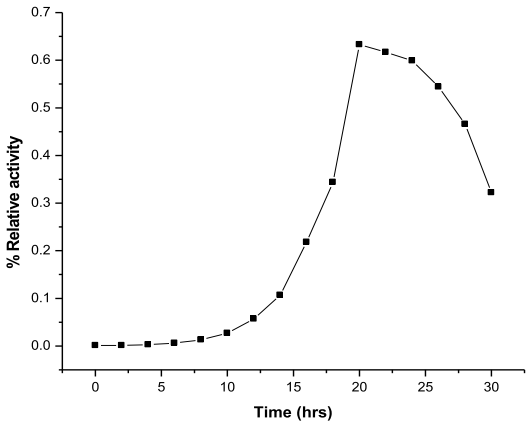

Fig. 3. Growth curve of *Rhodococcus sp*. LKE-021 (O.D. at 600 nm) at shake flask level

Molecular Identification of LKE-021

NCBI database was used for 16S rRNA sequence alignment using clustalW software, and the phylogenetic tree prepared by the TREEVIEW program. Sequenced16S rRNA gene containing 1425bp was submitted to NCBI GenBank database. Based on the nucleotide sequences of 16S rRNA gene the stain *Rhodococcus sp*. LKE-021 was classified as a new *Rhodococcussp* isolate. The taxonomic position is revealed in the phylogenetic tree (Fig. 6). The ribosomal protein homology as

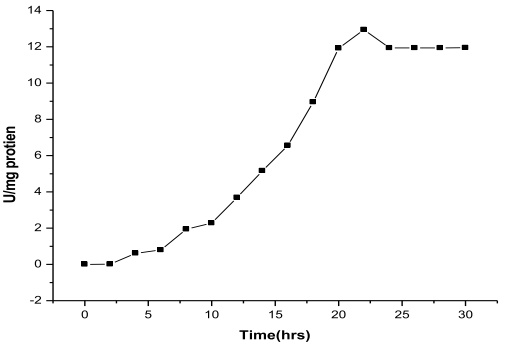

Fig. 4. Enzyme production of *Rhodococcus sp*. LKE-021 (U/mg) at shake flask level

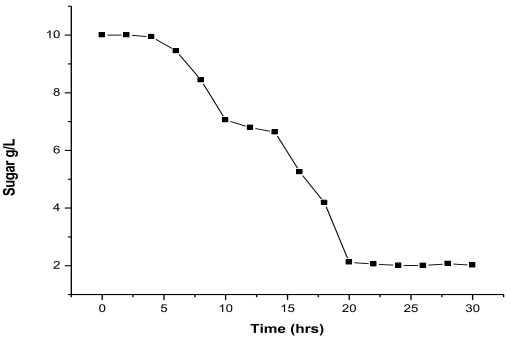

Fig. 5. Sugar utilization of LKE-021 (g/L) at shake flask level

Table 2. Alignment view using combination of NCBI GenBank and RDP database of strain *Rhodococcussp*LKE-021

Alignment View	RDP ID	Alignment Result	Sequence description
	LKE-021	0.97	**Studied Sample**
	X76690	0.99	Rhodococcus sp. str. PH114
	AJ131637	0.99	Rhodococcus erythropolis str. DCL14
	U87968	0.99	Rhodococcus X309 str. X309
	U82666	0.99	Nocardioides simplex ATCC 19565
	U82667	0.99	Nocardioides simplex ATCC 19566
	X79289	0.99	Rhodococcus erythropolis DSM 43066 (T)
	AF181691	0.99	Rhodococcus 7/1 str. 7/1
	AB010911	0.99	Rhodococcus sp. str. SRB1948-A07
	U81990	0.99	Nocardioides simplex ATCC 13260
	X76691	0.99	Rhodococcus erythropolis

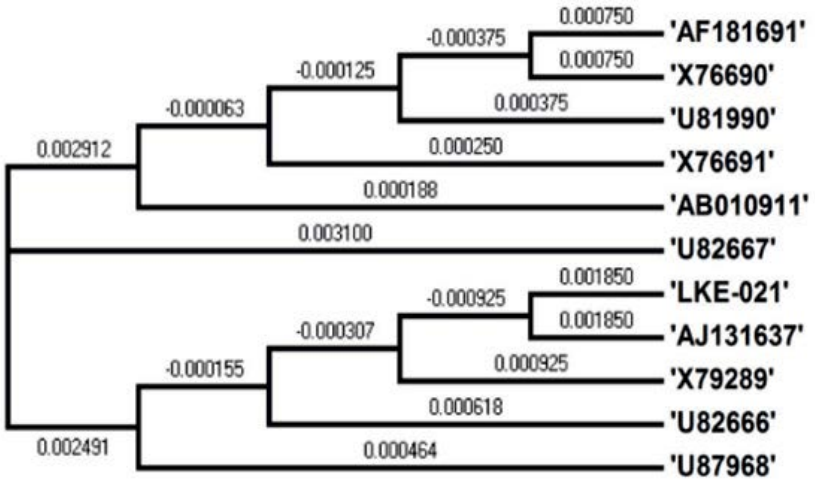

Fig. 6. Dendrogram indicating the position of the *Rhodococcus sp* LKE-021

well as similarity matrix was also reduced based on Nucleotide Sequence Homology of *Rhodococcus sp*. LKE-021 Using Kimura-2 Parameter. Alignment view via combination of RDP database and NCBI GenBank of *Rhodococcussp* LKE-021shown in Table 2. Nucleotide distance (below diagonal) and similarity (above diagonal) identities among the studied sample 'LKE-021' and ten other closest homologs microorganism indicates in Table 3.

Table 3. Distance Matrix of *Rhodococcussp* LKE-021based on Nucleotide Sequence Homology

Distance Matrix												
		1	2	3	4	5	6	7	8	9	10	11
LKE-021	1	---	0.990	0.996	0.990	0.996	0.990	0.996	0.996	0.990	0.996	0.989
AF181691	2	0.010	---	0.994	1.00	0.994	1.00	0.994	0.994	1.00	0.994	0.999
AJ131637	3	0.004	0.006	---	0.994	1.00	0.994	1.00	1.00	0.994	1.00	0.993
U81990	4	0.010	0.000	0.006	---	0.994	1.00	0.994	0.994	1.00	0.994	0.999
X79289	5	0.004	0.006	0.000	0.006	---	0.994	1.00	1.00	0.994	1.00	0.993
X76691	6	0.010	0.000	0.006	0.000	0.006	---	0.994	0.994	1.00	0.994	0.999
U82666	7	0.004	0.006	0.000	0.006	0.000	0.006	---	1.00	0.994	1.00	0.993
U87968	8	0.004	0.006	0.000	0.006	0.000	0.006	0.000	---	0.994	1.00	0.993
AB010911	9	0.010	0.000	0.006	0.000	0.006	0.000	0.006	0.006	---	0.994	0.999
U82667	10	0.004	0.006	0.000	0.006	0.000	0.006	0.000	0.000	0.006	---	0.993
X76690	11	0.011	0.002	0.007	0.002	0.007	0.002	0.007	0.007	0.002	0.007	---

DISCUSSION

Few of extremophilic esterase enzyme have been reportedfrom thermophiles, but there areinsufficient report of esterase enzyme activity from *Rhodococcus* sp.[21-23]reported. In current research work we are reporting the hyperthermo alkaline esterase enzyme isolated from *Rhodococcus* sp LKE-021, isolated from soil sample of Gangotri region of Himalaya Uttarakhand, India. Esterase production was optimized with nutrient brothfrom *Rhodococcus* sp LKE-021. Based on sequences of nucleotide 16s rRNA gene sequence of the strain found to be member of *Rhodococcus* sp LKE-021. LKE-021. Esterase studied by using various pNP and ethyle ester of straight chain of fatty acid and ranging of the chain from C_2 to C_{14}. Esterase showed specificity to substratelikep NP and ethyle ester. pNP and ethyle ester with acyle chain bigger length than C_8 were not suitable substrate for LKE-021.

Taxonomic position of the LKE-021 was showed in phylogenetic tree.

Kumar *et al.,* also isolated esterase from thermoalkaliphilic halotolerant *Rhodococcus sp*. LKE-028[12]. Known microbial enzymes are to play animportant role as metabolic catalysts, various uses in different industries and applications. The use market for industrial important enzymes is widely spread with various industrial commercial level applications. Many industrial products are made-up using enzymes. Many industrial processes, using chemical synthesis for pharmaceuticals, chemical, drug and food production have several disadvantages: devoid of enantiomeric specificity for chiral synthesis, low catalytic activity, need for low pH, high temperature and high pressure. Thermostable esterase are very important for the industrial process, such as drug industry, agricultural industry, food industry, cosmetic industry, pharmaceutical industry etc. Some thermostable esterase had been earlier reported such as thermophilic *Anoxybacillus gonensis*-A4 bacterium isolated from hot springs in Turkey[24], carboxyl esterase enzyme originated from *Bacillus subtilis* have used in the synthesis of naproxen for instance there isno steroidal anti-inflammatory drug[18] and 2-arylpropionic acids having high enantio-selectivity[25], *Bacillus coagulans*[26], *Bacillus stearothermophilus*[27]. Recently, diphenolases were studied from *Anoxybacillus kestanbolensis* strains K1 and K4T[28] as they all produced thermophile esterase. *Rhodococcus sp*. LKE-021 is batter then all strains because LKE-021 Esterase shows the activity on higher temperature *i.e.* 70°C and it is stable on 80-90°C.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

LS collected sample and isolation of *Rhodococcus* sp.. GS did DNA extraction and performed PCR. GA did 16S rDNA sequencing. LK did quantification and production of Enzyme. MIA checked grammer and formatted the manuscript. SM prepared Phylogenetic tree and analysed.

FUNDING

None.

DATA AVAILABILITY

All datasets obtained or studied during this study are incorporated in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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