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Research Article

ISOLATION AND CHARACTERIZATION OF BACTERIA FROM SOIL COLLECTED FROM HIMALAYAN REGION FOR THE PRODUCTION OF LIPASE BY SOLID STATE FERMENTATION USING TWEEN-20

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Abstract: Here we are reporting one microorganism isolated from soil collected from Himalayan region, producing thermostable lipase. Tween 20 agar, selective media was used to isolate soil microbe *Bacillus mojavensis* 5-SM. Molecular phylogeny of isolated strain shows similarity with *Bacillus mojavensis* 3EC4A5. Different parameters such as substrate selection, pH of the medium, temperature were optimized. Effects of various organic solvents, reducing and oxidizing agents, metal ions and surfactants were analyzed on extracted crude lipase. The maximum extracellular activity was observed at pH 11 and temperature 60^0C as optimum.
Key words: *Bacillus mojavensis* 5-SM, Crude lipase, Molecular phylogeny, Thermostable lipase.
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INTRODUCTION

Himalayan biosphere is a treasure house of microbial diversity which remained almost unexplored. Here we report an enzyme producing microorganism isolated from soil taken from hills of Dehradun region and through this dissertation work we targeted to isolate microbe which can produce lipase and successfully isolated, such microorganisms which can produce target enzyme and enzyme have been characterized in crude form. Through this report we propose that the microorganisms are ready for bioprocessing experiments in a laboratory scale fermentation which could not be done due to short tenure of project time. Soil microorganisms have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with novel enzymes. So, we have reported lipase produced by soil bacteria that is, *Bacillus mojavensis 5-SM.*

MATERIALS AND METHODS

Soil was collected from Himalayan range using sterile spatula in sterile glass tubes having plastic caps and after collection; the cap was sealed with *Parafilm*. After serial dilution pure culture was obtained by streaking the culture in nutrient agar media in sterilized conditions. It was then screened for its lipolytic activity on Tween 20 agar solid medium at 35^0C for 3 days, which was done to bright clear zone or hydrolytic zone on the plate. After general identification through gram's staining we went through with molecular identification of 5-SM; outside laboratory bacterial identification based on 16S rDNA data and other extracellular enzyme characterization *(Chaturvedi et al., 2010).*

Optimization of enzyme production: Tween-20 broth was inoculated with 5-SM and incubated in 35^0C at 120 rpm. Samples were collected after 0, 24, 48 and 72 hr. and all the parameters like growth profile, enzyme production were estimated in order to optimize standard enzyme production condition and time using shaking waterbath and incubator. Proper microbial growth helps in expressing extracellular enzymes and so growth profile of the 5-SM was monitored by turbidimetric method. Each aliquot was spectrophotometrically analyzed at λ_{660}. All the readings were analyzed as percentage relative activity of enzyme. Quantitatively lipase activity was analyzed spectrophotometrically at 660nm for which 100µg per ml solution of Tween 20 with enzyme extract and water was prepared. Incubate the mixture at 35^oC for 20 minutes. 10% stock TCA solution was prepared and took 500 µl of TCA and added to reaction voil. Mixture was centrifuged at 7000 rpm for 8 minutes. Read O.D. at 660nm.

Characterization of 5-SM Lipase

Effect of pH on activity and stability of extracellular enzyme: The pH optima for 5-SM lipase was determined using the following buffers: 0.1M citrate buffer (pH 5.0–6.0), 0.1M Tris-chloride buffer (pH 7.0–9.0), 0.1M Glycine – NaOH buffer (pH 10.0–11.0), 0.1M Na_2HPO_4–NaOH buffer (pH 12.0). Enzyme reactions in each buffer were carried out at 35 ^{0}C and reaction of lipase was stopped by DNS; their residual activities were measured periodically at 660 nm spectrophotometrically. *(Kumar et al., 2011)*

Optimum temperature of extracellular 5-SM lipase: The temperature optimum assay was performed for different temperatures in the range of 30-80^{0}C at the optimum pH value. The reaction mixtures were incubated for 15 min at various temperature ranges. Then, the relative activity was determined spectrophotometrically at 660 nm as rapidly as possible. *(Kumar et al., 2011)*

Effect of metal ions on activity of extracellular enzyme: The enzyme was incubated at optimum temperature for 1 h in optimum pH buffer, containing different metal ions, Ni^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+} and Zn^{2+} (final concentration of 5 mM). Residual activity was measured following standard assay method with Tween 20 as substrate and using DNS to stop reaction. Activity of the enzyme treated in the same way but without any additive was taken to be 100%. *(Kumar et al., 2011)*

Effect of organic solvents on activity and stability of the enzyme: In different screw cap test tubes, 2.0 ml of the enzyme solution in glycine- NaOH buffer (pH 11) was mixed with 1 ml of different organic solvents (poly ethylene glycol, xylene, butanol, ethanol, iso-propyl alcohol (IPA), propanol, petroleum ether and glycerol) and incubated at 35^{0}C with constant shaking for 2 days. Residual activity of an aliquot was measured by the standard assay both days. Loss of the solvents due to evaporation was replenished with fresh solvent. Appropriate control was included to eliminate the changes in the extinction coefficients due to presence of solvents. Spectrophotometric readings were taken at 660nm.

Effect of surfactants, oxidizing and reducing agents on enzyme activity: The enzyme was incubated at 60 ^{0}C for 1 h in Glycine- NaOH buffer (pH 11), containing one of the different chemical reagents including surfactants, oxidizing and reducing agents. To investigate the effect of oxidizing and reducing agents on enzyme activity, both types of agents (β-mercaptoethanol as reducing agent and hydrogen peroxide as oxidizing agent) were added to the enzyme and buffer mixture at a final concentration of 5 mM. The surfactants (Triton X-100, Tween 40) were added at a final concentration of 1 and 2% (v/v). Residual activity was measured following standard assay method with Tween 20 as substrate. Activity of the enzyme treated in the same way but without any additive was taken to be 100%. *(Kumar et al., 2011)*

RESULTS AND DISCUSSION

Primary Screening of Lipase producing microorganisms based on qualitative test shows clear hydrolytic zone *(Fig-1).*

Identification of 5-SM

The morphological and physiological characteristics of the isolate 5-SM was observed as Gram negative, rod shaped. Taxonomic characterization was done based on the nucleotide sequence of the 16S rRNA gene. On the basis of 1425 bp of 16S rRNA gene was sequenced and submitted to NCBI GenBank (accession no. EU304930). Based on the nucleotide sequence of 16S rRNA gene the isolate was classified as an existing *Bacillus mojavensis* 3EC4A5. The taxonomic position is shown in the phylogenetic tree *(fig-2)*. The ribosomal protein homology as well as similarity matrix was also deduced based on Nucleotide Sequence Homology of 5-SM. *Table-1* indicates nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample and twenty other closest homolog microbes.

Study of growth profile: Maximum growth of culture observed at 72 hrs *(Graph-1).*

Quantitative assay of lipase activity: Maximum lipase activity also observed at 72 hrs (*Graph-2).*

Characterization of 5-SM Lipase

Effect of pH on activity and stability of extracellular enzyme: The optimum pH for the activity of the enzyme is 11.0 and it is active over a broad range of pH 5.0–12.0 *(Graph-3).*

Optimum temperature of extracellular 5-SM lipase: The extracellular enzyme shows quite good activity over a temperature range of 30-80^{0}C, optimum activity being at 60 ^{0}C as evident from *Graph-4*. The thermal stability profile of 5-SM lipase in the form of residual activity was shown in graph. The enzyme was stimulated by keeping at 30-80^{0}C and almost retained its optimum activity at 60^{0}C for 2 hr incubation period.

Effect of metal ions on activity of purified enzyme: All metal ions studied enhance extracellular lipase activity to some extent. The effect of different metal ions on the lipase activity is shown in *Graph-5.*

Effect of surfactant, reducing and oxidizing agents on activity and stability of the enzyme: In presence of all surfactants like Tween 80 and triton (1% and 2%) demonstrated enhancement of the enzyme activity (*Graph-6).* In all cases the enzyme was subjected to 1 h of pre incubation. 5-SM lipase is stable in both oxidizing and reducing environment and retains its activity in the presence of β-mercapto-ethanol (β-ME) and hydrogen peroxide (*Graph-7).*

Effect of organic solvents on activity and stability of the enzyme: The purified 5-SM lipase showed higher activity with xylene, butanol and petroleum ether on first day results. Second day spectroscopic results showed reduced activity of lipase with all organic solvents used at 35^{0}C. As evident in graph, this enzyme is not only stable in some organic solvents, but also, the activity increases in the presence of organic solvent as compared to that in aqueous buffer at first. A dramatic decrease has been observed in second day O.D. (*Graph-8*).

Fig-1 5-SM bacteria showing hydrolytic activity in surrounding

Table 1: Alignment view using combination of NCBI GenBank and RDP database

S.No.	Similarity Score	Seqmatch score (S_ab)	Unique Common Oligomers	NCBI/ RDP ID	Sequence Description
1	0.993	0.951	1138	EU304929	Bacillus licheniformis3EC4A4
2	0.995	0.957	1144	EU304930	Bacillus mojavensis3EC4A5
3	0.995	0.958	1136	EU304931	Bacillus mojavensis3EC4A6
4	0.992	0.947	1212	EU304940	Bacillus licheniformis3EC4A15
5	0.993	0.952	1091	EU304950	Bacillus licheniformis3EC4B9
6	0.992	0.957	1277	EU334005	Bacillus licheniformisYDGW5
7	0.993	0.949	1326	AM950310	Bacillus sp. US2 HS-2008
8	0.993	0.949	1326	AM950314	Bacillus sp. US4 HS-2008
9	0.993	0.951	1319	EU770587	Bacillus licheniformisSVD1
10	0.992	0.947	1353	FJ458450	Bacillus licheniformisycsd02
11	0.989	0.947	1314	AB523710	Bacillus licheniformisAN-S10
12	0.988	0.947	1314	AB523711	Bacillus licheniformisAN-S16
13	0.987	0.947	1318	AB523712	Bacillus licheniformisAN-sR16
14	0.987	0.947	1318	AB523713	Bacillus licheniformisAN-sR21-1
15	0.989	0.947	1314	AB523714	Bacillus licheniformisAN-sR21-2
16	0.989	0.947	1314	AB523715	Bacillus licheniformisAN-sR34
17	0.989	0.947	1314	AB523718	Bacillus licheniformisAN-sS8-2
18	0.988	0.948	1315	AB523719	Bacillus licheniformisAN-sS23
19	0.989	0.947	1314	AB523720	Bacillus licheniformisAN-sB
20	0.99	0.955	1309	HQ202723	Bacillus licheniformisP-104

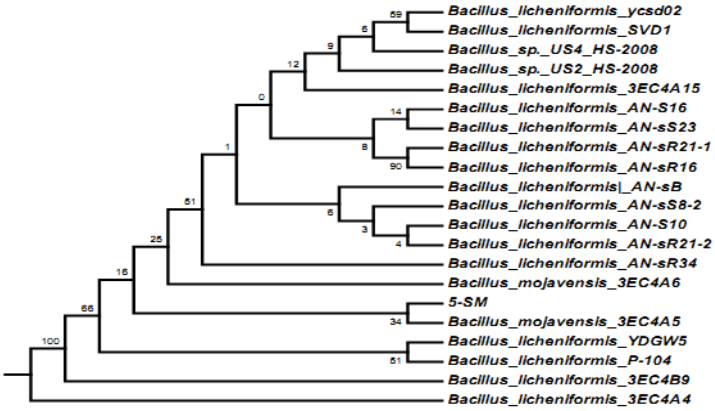


Fig-2: Phylogenetic Tree (NJ method)

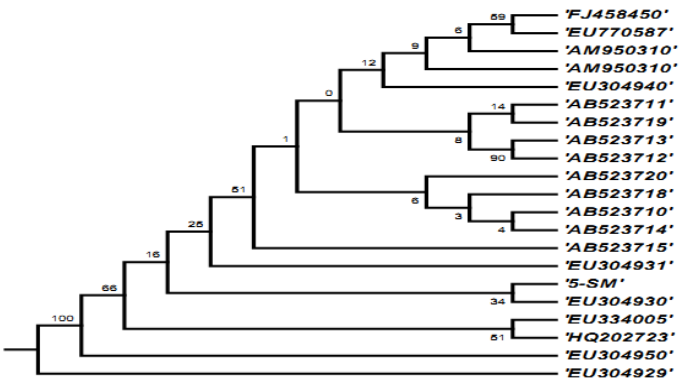
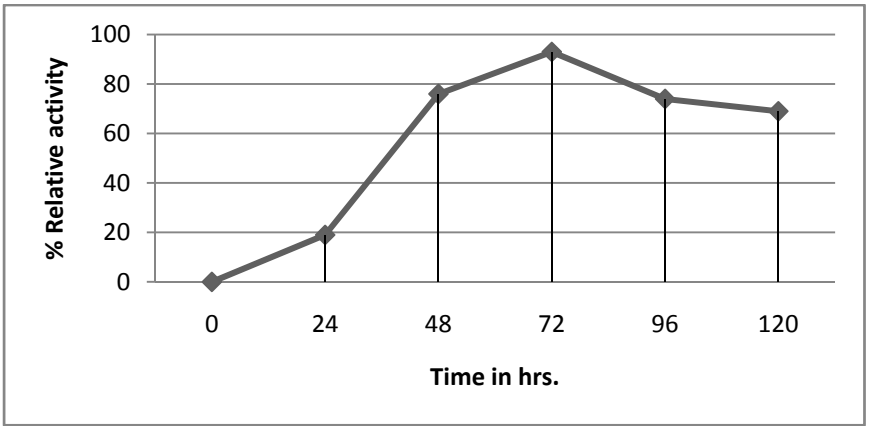
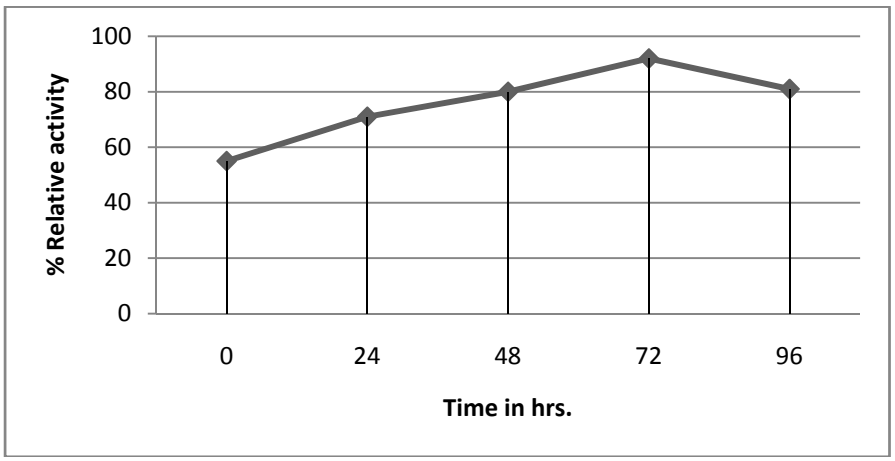


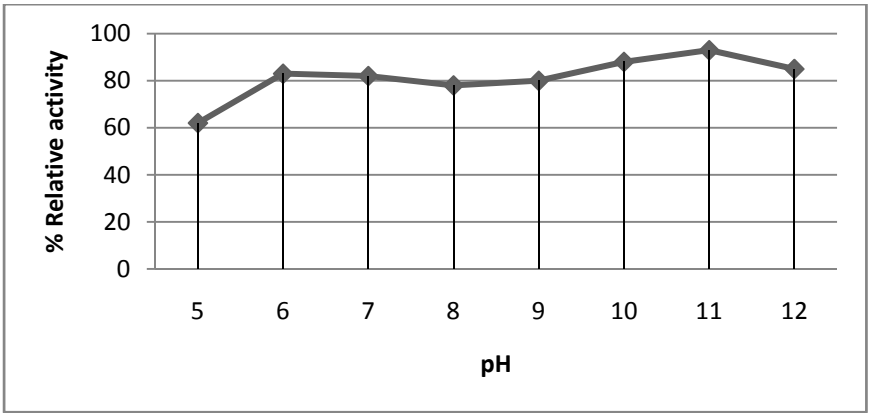
Fig-3: Phylogenetic dendrogram position of *Bacillus mojavensis 5-SM*



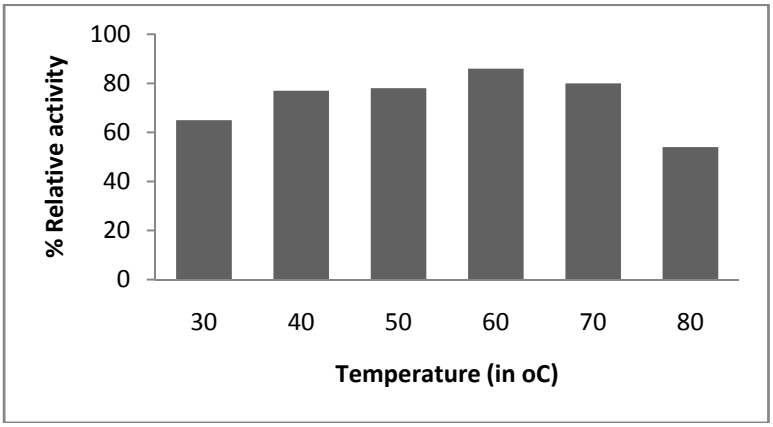
Graph-1: Study of growth profile



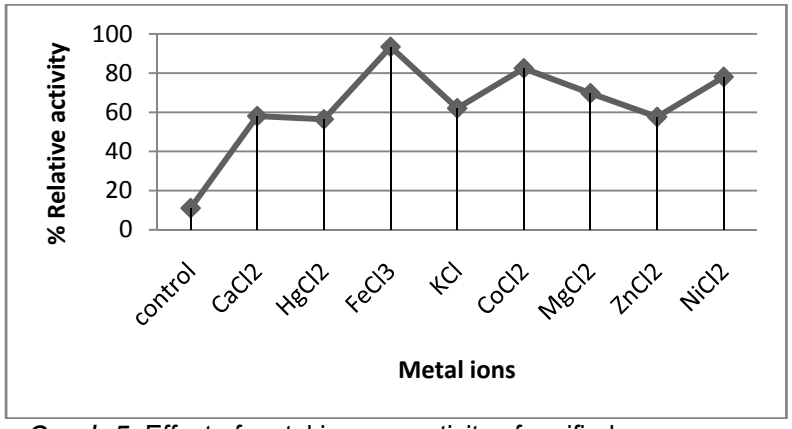
Graph-2: Quantitative assay of lipase activity



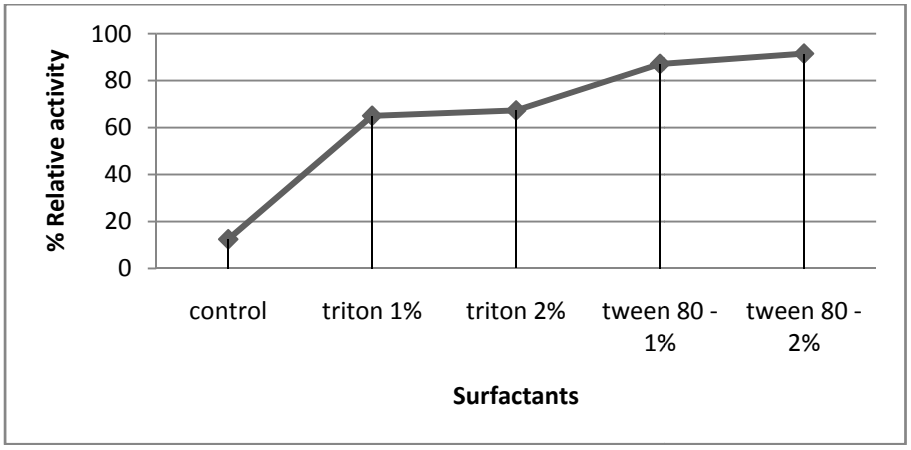
Graph-3: Effect of pH on activity and stability of extracellular enzyme



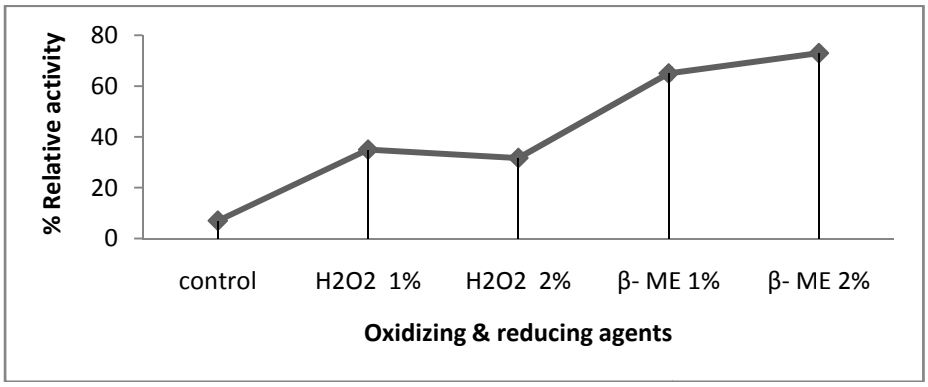
Graph-4: Optimum temperature of extracellular 5-SM lipase



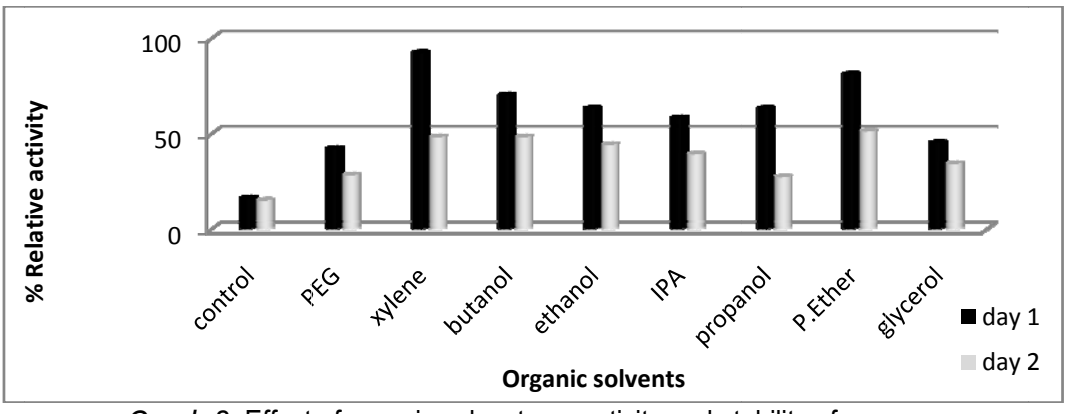
Graph-5: Effect of metal ions on activity of purified enzyme



Graph-6: Effect of surfactant on activity and stability of the enzyme



Graph-7: Effect of reducing and oxidizing agents on activity and stability of enzyme



Graph-8: Effect of organic solvents on activity and stability of enzyme

CONCLUSION

5-SM lipase enzyme produced by the soil bacteria *Bacillus mojavensis* 5-SM sps. It can be summarized that the enzyme of 5-SM is more suitable for further use at industrial level. 5-SM showed a promising activity at 60^0C and stable at 30-80^0C however its higher stability shows that our strain is highly thermostable. In case of pH study enzyme activity was found to be more active up to 5-11 (optimum pH 11). Optimum growth conditions for *Bacillus mojavensis* 5-SM indicates its alkaline-thermostable nature and since enzyme. The 5-SM lipase showed unstability in the presence of organic solvents as the results were shown. A drastic fall occurs in organic solvents activity on enzyme on second day results. Enzyme showed stability with surfactants, oxidizing and reducing agents like β mercaptoethanol, hydrogen peroxide, triton X, Tween 80. Effect of Zn metal impacts more over other

metals, suggests increased enzyme activity. Characterization studies of extracted lipase shows its potent use at Pharma, Food, Enzyme industries.

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