ESTIMATION OF EXTRACELLULAR LIPASE ENZYME PRODUCED BY THERMOPHILIC *BACILLUS* SP. ISOLATED FROM ARID AND SEMI-ARID REGION OF RAJASTHAN, INDIA

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ABSTRACT

Thermophilic organisms can be defined as, microorganisms which are adapted to live at high temperatures. The enzymes produce by thermophilic bacteria capable of catalyzing biochemical reactions at high temperatures. Thermophilic bacteria are able produce thermostable lipase enzymes capable of degradation of lipid at temperatures higher than those of mesophilic bacteria. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are quite useful in terms of discovering thermophilic lipase enzymes. Due to great temperature fluctuation in hot arid and semi-arid region of Rajasthan, this area could serve as a good source for new thermophilic lipase producing bacteria with novel industrially important properties. The main objective of this research is the isolation and estimation of industrially important thermophilic lipase enzyme produced by thermophilic bacteria, isolated from arid and semi-arid region of Rajasthan. For this research purpose soil samples were collected from Churu, Sikar and Jhunjunu regions of Rajasthan. Total 16 bacterial strains were isolated and among all these bacterial isolates only 2 thermophilic lipase producing bacteria were identified. The thermophilic lipase enzyme was estimated by qualitative and quantitative experiments. The isolate was identified as *Bacillus* sp. by microscopic, biochemical and molecular characterization. The optimum enzyme activity was observed at pH 8, temperature 60°C and 5% salt concentration at 24 hrs time duration. Lipases find useful in a variety of biotechnological fields such as food and dairy (cheese ripening, flavour development), detergent, pharmaceutical (naproxen, ibuprofen), agrochemical (insecticide, pesticide) and oleochemical (fat and oil hydrolysis, biosurfactant synthesis) industries. Lipases can be further use in many newer areas where they can serve as potential biocatalysts.

MATERIAL & METHOD

Soil samples were collected in sterile containers from the hot regions of Rajasthan, India.
The temperature of the soil was between 50 °C to 55 °C and the pH of the soil was around 7.5.
The collected soil samples were serially diluted up to 10^{-1} to 10^{-4} and spreaded on agar plates and incubated at 50 °C.

Screening for lipase producing bacteria:
Screening of lipase producers was carried out using tributyrin agar plates (Lawrence 1967).
Each culture was streaked onto the tributyrin agar plate and incubated at 50°C for 2 days.
The lipase producing bacteria were identified by the presence of clear hydrolytic zones.

Identification of lipase producing bacteria:

Cultural characterization
The isolates were observed under the microscope, the bacterial colony morphology was noted with respect to color, size, shape, nature of colony and pigmentation.

Microscopic observation
For microscopic observation of bacterial isolates, these bacterial strains were Gram stained , Endospore stainied, further capsule staining and motility test were performed to observe the morphology and motility of the cells.

Biochemical characterization
Biochemical characterization of bacterial isolates were performed by indole test, methyl red test, voges proskauer test, Simmons citrate test, Starch hydrolysis test, H_2S production, catalase test, oxidase test, urease test, nitrate reduction test, gelatin hydrolysis test.

Molecular characterization by DNA preparation and PCR amplification
Genomic DNA was extracted from the isolates using CTAB method. Each genomic DNA used as template was amplified by PCR with the aid of 16S rDNA primers (16S Forward Primer: 5'- TGCGGCTGGATCCCCTCCTT-3', 16S Reverse Primer: 5'-CCGGGTTTCCCCATTCGG-3') and thermal cycler was programmed as denaturation at 94°C for 2 min followed by subsequent 30 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 45 sec, extension at 72°C for 45 with the final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10µl of the reaction product in 1% agarose gel.

16S rDNA sequencing and data analysis:
Sequencing analysis was performed on a 1500 bp PCR product.
The sequence analysis was performed using the ABI automated sequencer.
The two 16SrDNA sequences were aligned and compared with other 16SrDNA genes in the GenBank by using the NCBI Basic Local alignment search tools BLAST.

Enzyme Production:
The identified bacterial lipase producers were cultivated in production medium (50 ml) in triplicate (one control and two replicates / sample).
After incubation at 50°C for 36h the cultures were centrifuged at 8000 rpm at 4°C for 15 min. The crude lipase solution was obtained by filtering through a 0.22µm pore size membrane filter and was used as the source of crude lipase enzyme.

Effect of Temperature, NaCl, Time period and pH on the Activity of Lipase:
The effect of temperature on catalytic activity of lipases was determined by measuring the enzyme activity at temperature range from 40°C- 65°C under the standard assay conditions (fig4).
The effect of pH on enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 3 to 9 at 60 ºC using suitable buffers (fig 5).
Effect of NaCl on enzymatic activity was measured by using range 1 to 7% NaCl finally effect of time duration also measured by estimated enzyme quantity at different time (24h, 48h, 72h, 96h and 120h).

RESULT

- Total ten soil samples were collected from different regions of Rajasthan.
- Both dilution plate and streak plate method were used for isolation of thermophilic lipase producing bacteria.
- Total bacterial strains were isolated and those bacterial strains showing extracellular lipase enzyme activities were selected for further analysis.
- Two different strains showing clear hydrolytic zone on Tributyrin agar (fig1).

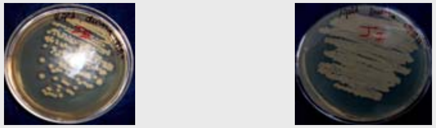


Fig.1: Clear zone indicates the hydrolysis of Tributyrin as a result of lipase production

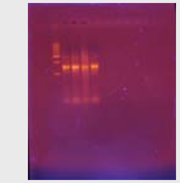
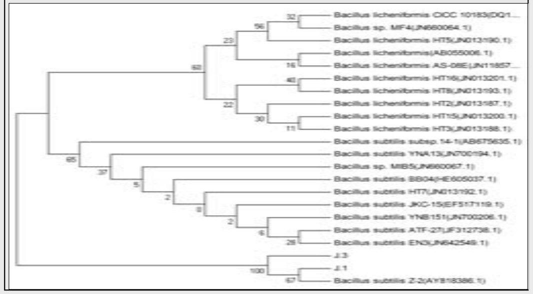


Fig. 2: 16SrDNA PCR amplification of bacterial isolates

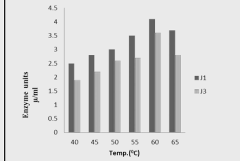
Mode of characterization	Characteristics of bacterial isolates	J1 bacterial strain	J3 bacterial strain
Cultural characteristics .	Colony morphology on nutrient agar	Irregular, mucoid, creamy yellow, fast growing colonies	Small, round, regular, mucoid, creamy yellow, fast growing colonies
Microscopic characteristics	Spore staining Gram staining Motility	Spore forming Gram positive	Spore forming Gram positive motile
Biochemical characteristics	Indole test Methyl Red test Voges Proskauer test Citrate utilization test Catalase test Oxidase test Urease test Nitrate reduction test Gelatin liquefaction test Starch hydrolysis test Hydrogen sulphide test Hydrogen peroxidase test Casein hydrolysis test Glucose fermentation	Negative Negative Positive Positive Positive Positive Negative Positive Positive Positive Negative Positive Positive Negative	Negative Negative Positive Positive Positive Positive Negative Positive Positive Positive Negative Positive Positive Negative

Table 1: Biochemical Identification of the lipase Producing Thermophilic bacteria

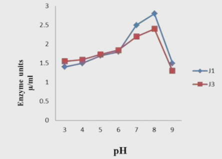
PHYLOGENETIC TREE OF *BACILLUS* SP. J1 AND J3



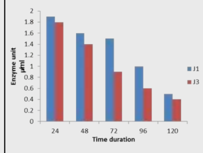
EFFECT OF TEMPERATURE, pH, TIME DURATION & NaCl CONCENTRATION ON LIPASE ACTIVITY & QUANTITY



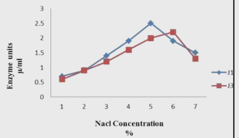
Effect of Temperature on the enzyme activity



Effect of pH on the lipase activity



Effect of time duration on production of the lipase activity



Effect of NaCl concentration on production of the lipase activity

CONCLUSION

- Known industrial applications of thermostable lipases include, production of mono- and diacylglycerides, fatty acids and glycerol through hydrolysis of oils and fats, modification of the composition or physical properties of triacylglycerides, synthesis of chemicals in organic solvents, paper manufacturing, biochemical catalyzer in supercritical fluids (Markossian *et al.,* 2000) and waste sludge can be treated at temperatures above 60^0C (Markossian *et al.,* 2000).
- This study reports biological production of thermostable lipase by soil bacteria *B. Licheniformis* and *B. subtilis*. Enzyme production was find maximum in presence of 5% NaCl concentration, temperature 60°C and pH 8.0.
- At the optimum conditions *B. Licheniformis* or *B. subtilis* produced higher amount of enzyme than before optimization on the other hand these bacterial species producing thermostable lipase enzyme which shows catalytic activity at high temperature that is not possible for mesophilic bacteria .

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