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WHOLE CELL IMMOBILIZATION BY ENTRAPMENT METHOD FOR LIPASE PRODUCTION

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ABSTRACT

Enzyme immobilization using biopolymers like sodium alginate enhances their stability and reusability, making them valuable in biotechnology. Soil samples from oil-contaminated sites in Surat, India, were screened on Tween 80-supplemented nutrient agar. A potent lipase producer, VC 13, was selected and characterized by morphological, biochemical, and molecularly characteristics and identified Cytobacillus sp. And its phylogenetic analysis was carried out. Whole-cell immobilization was done using 1.5% sodium alginate, forming $CaCl_2$-stabilized beads. Parameters such as temperature, pH, biomass, alginate concentration, and storage stability were optimized. Immobilized cells showed good enzyme activity, with optimal performance at 37°C, pH 8.5, 100 mg biomass, and 3% alginate. The enzyme retained activity over extended storage, suggesting its potential for industrial use.

INTRODUCTION

Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, soil contaminated with oil, oilseeds, vegetable oil processing factories, dairies, and decaying food. Lipases derived from microbial sources were first identified early in the 20th century (Jaeger et al., 1994). Until recent years, metagenomics lipases and artificially designed lipases have gradually appeared in people's vision (Tang et al., 2017; Cen et al., 2019; Almeida et al., 2020; Verma et al., 2021). For lipase producing bacteria the selective media is use which contains substrates like olive oil, Tween 80, tributyrin or Rhodamine B agar. A variety of extracellular lipases of bacterial origin with different properties has been described. Extracellular lipases have been produced from microorganisms, such as fungi, yeast and bacteria, beside from plants, and animals: whereas commercial lipases have been produced from *Pseudomonas cepacia*, *Pseudomonas alcaligenes* and *Pseudomonas mendocina* (Chigusa et al., 1996). Lipases are a group of enzymes that catalyze the hydrolysis of ester bonds in lipids, establishing their necessity in various biological processes, different industrial applications, and medical treatments. Lipases are a class of enzymes that catalyze the hydrolysis of ester bonds in triglycerides and phospholipids, breaking them down into glycerol and free fatty acids.

The immobilization of lipase refers to the process of attaching lipase enzymes to solid support while retaining their catalytic activity. This technique amplifies the enzyme's stability or reusability. Alginate is most widely used polymer for immobilization and microencapsulation technologies. The main advantage in alginate gels is their thermostability, and thus they can be stored in room temperature.

MATERIALS AND METHODS

Isolation and Screening of Lipase-Producing Bacteria

Soil samples contaminated with oil were collected from different places were serially diluted (10^{-1} to 10^{-6}) and 0.1 ml aliquots from each dilution were plated in nutrient agar supplemented with 1% tween-80. (Gupta et al.,2012) The plates were incubated at 30°C for 48 hrs. Screening of lipase producer were carried out by determining visual precipitation of calcium salts caused by the fatty acids released from the hydrolysis reaction. Selected isolates were maintained on the same medium for future use. (Van Hong Thi Pham et al., 2021).

Characterization of Isolates

Morphological tests were performed to observe the growth patterns of microorganisms i.e., motility test, gram staining and various biochemical tests (Indole test, Methyl red (M-R) test, Voges-Proskauer (VP) test, Hydrogen sulphide (H2S) production test, Nitrate reduction test, Citrate utilization test, Urea hydrolysis test, Gelatin liquefaction, Catalase test (slide test), Oxidase test, Sugar utilization test and Triple Sugar Iron (TSI) agar slant) were performed using a standard protocol to know the diversity of isolate followed by Bergey's manual. (Cossart, 1987).

Molecular identification of selected isolate was carried out by extracting genomic DNA. Unidirectional partial sequence of 16S rRNA of bacterial isolates were amplified using 907R – 5'-CCGTCAATTCGGTGA-3' as reverse primer. Identification of isolates were carried out by using BLAST (Basic Local Alignment Search Tool) with the database of NCBI (National Centre for Biotechnology Information) gene bank database based on maximum identity score.

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Phylogenetic Tree Analysis of Selected Isolate

The phylogenetic analysis of selected isolate was carried out on the basis of partial 16S rRNA sequence the nucleotide sequence of related organisms was obtained from the NCBI database and used for alignment and calculating the homology level. ClustalW programme was used to align the sequences. The Phylogenetic tree was constructed by the neighbor - joining method using MEGA 11 (Molecular Evolutionary Genetics Analysis) software. Partial sequence of 16S rRNA of R1 was deposited in NCBI database using BankIt submission tool of Gene bank. (Tamura K. et.al., 2021).

Lipase Assay by Titrimetric Method

The lipase activity was assayed according to Kimura et al. (7) with slight modifications. Culture filtrate (1 ml) was shaken with 2.5 ml olive oil emulsion in water (1:1, v/v) and 20 µl of 0.02 M $CaCl_2$ solution in a water bath shaker at an agitation rate of 200 rpm. The reaction mixture was shaken for 30 min at 37°C. Reaction was stopped by adding 7.0 ml of acetone-ethanol (1:1, v/v) $_{mixture}$ and was then titrated against 0.02 M NaOH solution to pH 10. Unit activity is expressed as µmol of fatty acids liberated per min. (Zainoh Zakaria et al., 1992).

Whole cell Immobilization of Lipase Producing Microorganism by Entrapment Method

The cells were entrapped in Sterile Na-alginate beads by mixing 50mg lipase cell biomass (100 units) with 10mL of sterile sodium alginate solution (1.5% w/v). The mixture was stirred and dripped through a sterile pipette into 10mL of sterile 2% $CaCl_2$ solution. After 30min of hardening in the same solution, the beads were separated from the sterile $CaCl_2$ solution by filter paper and the beads were inoculated in sterile nutrient broth supplemented with 1% tween-80, incubated at 30°C for 24 hrs. After 24 hrs, Beads were washed on a filter with 1% $CaCl_2$ and then with 0.05M Tris buffer pH 7.5, so as to remove the loosely bounded enzyme. The filtered $CaCl_2$ solution and washings were collected and tested for lipase activity to determine the binding efficiency. (Indu Bhushanet al., 2008).

Optimization of Parameters for Whole Cell Immobilization

1. Effect of Incubation Temperature

The beads were incubated at different temperature (10°C, 20°C, 30°C, 37°C and 45°C) to check optimize temperature for lipase production. After incubation, filtrate and washing solution were collected and tested for lipase activity (Indu Bhushanet et. al., 2008).

2. Effect of washing buffer pH

The pH of the washing solution of tris buffer were changed (5.5, 7.5, 8.5 and 9.5) to check optimize pH for lipase production. After washing with different pH, filtrate and washing solution were collected and tested for lipase activity (Indu Bhushanet et. al., 2008).

3. Effect of Biomass

The various cell biomass were collected (50mg, 100mg and 200mg) to make beads for lipase production. After incubation, filtrate and washing solution were collected and tested for lipase activity (Zainoh Zakaria et al., 1992).

4. Concentration of Sodium Alginate

1.5%, 3% and 5% sodium alginate solution were used for immobilization of cell to check its binding efficiency. Further, filtrate and washing solution were collected and tested for lipase activity (Zainoh Zakaria et al., 1992).

5. Storage Stability

The beads were stored in refrigerator and the lipase activity were checked at the interval of 5 days (Indu Bhushanet et. al., 2008).

RESULTS AND DISSCUSSION

Isolation and Screening of Lipase-Producing Bacteria:

Amongst the 30 isolated colony, Isolate VC 13 was selected for further study due to its highest lipase activity (2.66 Units/ml). The result of biochemical characterization of VC 13 shown in Table 1

Table 1 Result of biochemical test

Biochemical test	Result	Biochemical test	Result
Indole test	-	Nitrate reduction test	-
MR test	-	Urea hydrolysis test	-
VP test	-	Gelatin liquefaction test	-
Citrate utilization test	+	Catalase test	+
H_2S production test	-	Oxidase test	+
Sugar fermentation test		**TSI agar slant**	
Nutrient sucrose broth	+	Slant	A
Nutrient xylose broth	+	Butt	Al
Nutrient mannitol broth	+	CO_2 Production	-
Nutrient maltose broth	+	H_2S Production	-
Nutrient lactose broth	-		

(**A**= Acid, **Al**= Alkaline, + = Positive test, - = Negative test)

Isolate VC 13 was further identified by performing the 16S rRNA sequencing by using Sanger dideoxy sequencing method, the selected isolate number VC 13 obtained from the oily soil sample and it was identified as *Cytobacillus sp*. by using BLAST tool (Basic Local Alignment Search tool) of NCBI (National Center for Biotechnology Information). The partial sequence of 16S rRNA of *Cytobacillus*

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sp. Is 769 nucleotide long. This partial sequence of *Cytobacillus sp.* (VC 13) has been submitted to NCBI having accession number PV522821.

Phylogenetic tree analysis *Cytobacillus sp.* VC 13

The phylogenetic tree was constructed for isolate VC 13 using MEGA software (version 11). Evolutionary tree analysis of isolate VC 13 along with set of organisms or group of organisms (taxa) was shown in Figure 34. Evolutionary history was inferred using the Neighbor-Joining method (Saitou N. and Nei M., 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J.,1985) The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K., Nei M., and Kumar S., 2004) and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1481 positions in the final dataset. Evolutionary analyses were conducted in MEGA11(Tamura K., Stecher G., and Kumar S., 2021)

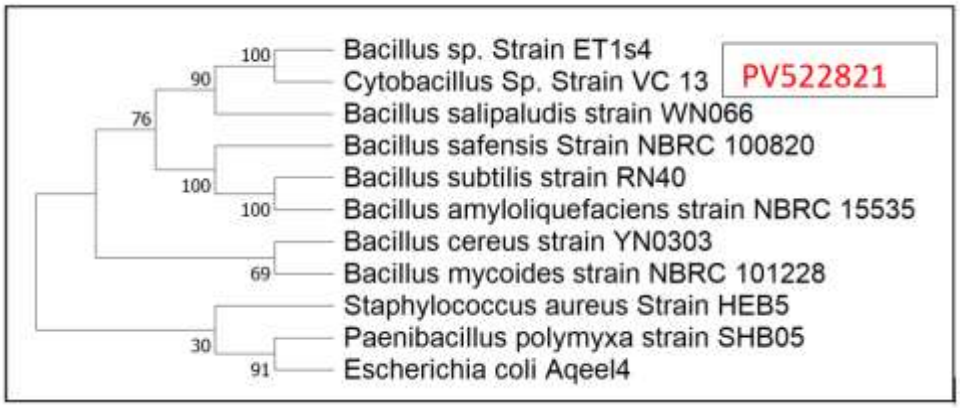


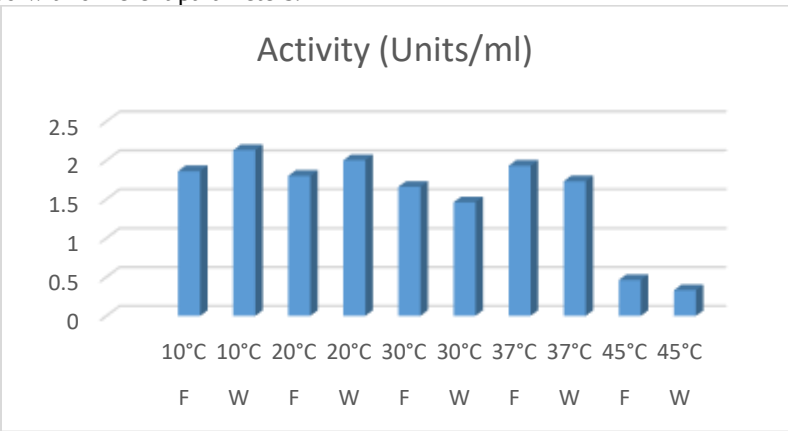
Figure 1 Figure Phylogenetic tree of *Cytobacillus sp.* VC 13.

Whole Cell Immobilization and Optimization of lipase producing organism

The organism which gives highest lipase activity *Cytobacillus sp.* (VC 13) cells were immobilized in nutrient agar supplemented with 1% tween-80. Further, lipase activity of particular organism was tested by titrimetric method with different parameters.

Effect of Incubation Temperature

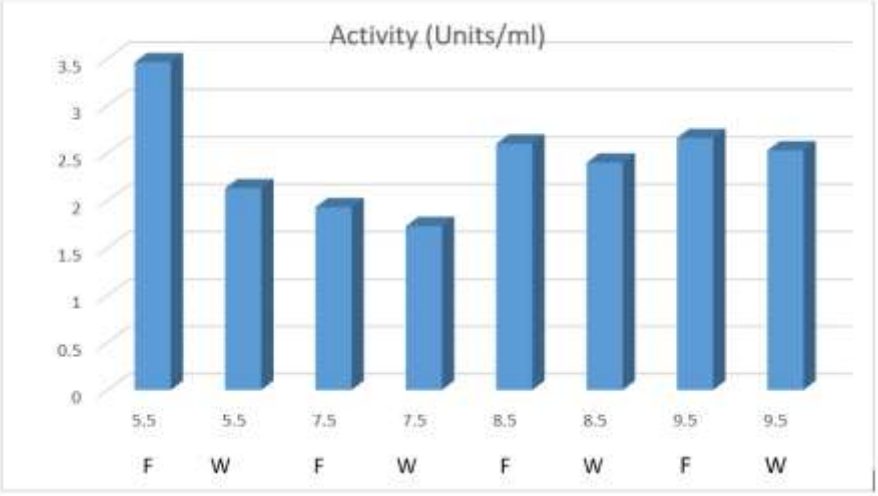
The immobilized beads were incubated at different temperature to check the lipase production. After incubation the highest activity was observed at 37°C (Graph 1).



Graph 1: Effect of temperature. (F – Filtrate, W- Wash solution)

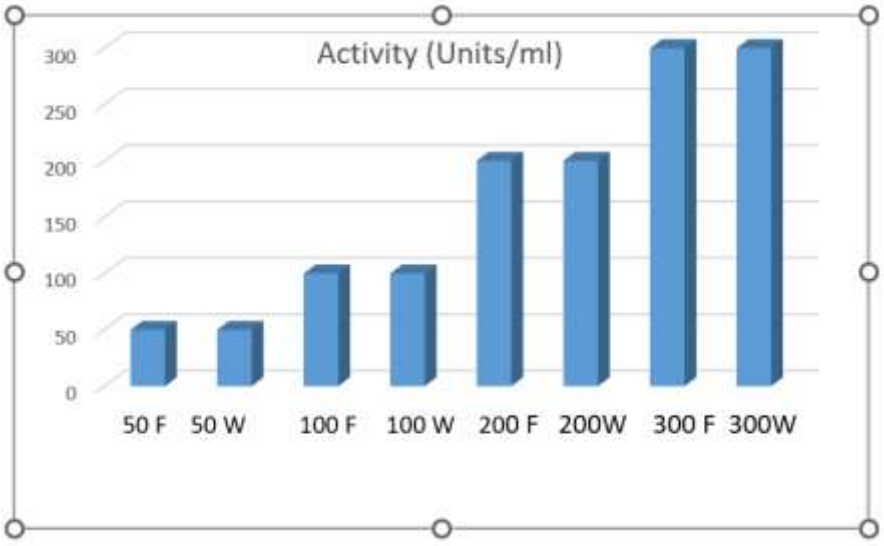
Effect of Washing Buffer pH: By changing the pH of tris buffer for washing immobilized beads the highest production was observed on pH 5.5 (Graph 2).

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Graph 2: Effect of washing buffer pH (F – Filtrate, W- Wash solution)

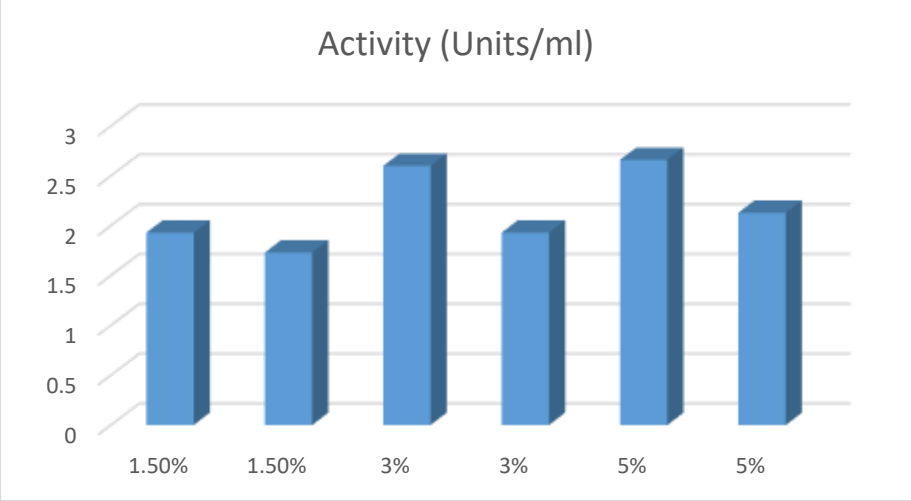
Effect of Biomass: Biomass concentration was varied for immobilization like 50, 100, 200 and 300mg. The highest activity was observed in 50 mg concentration. (Graph 3)



Graph 3: Effect of biomass concentration (F – Filtrate, W- Wash solution)

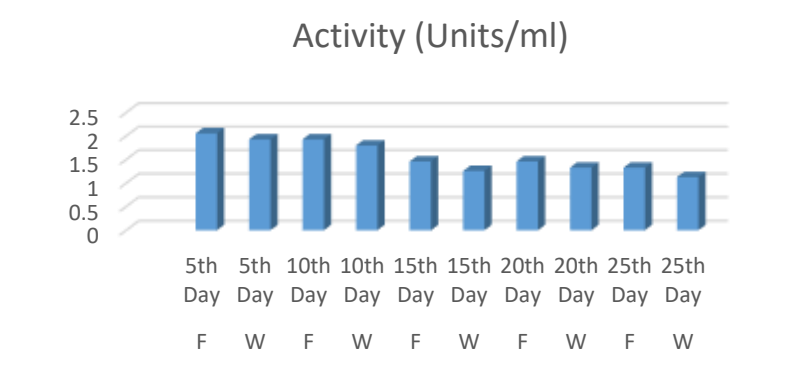
Effect of Sodium Alginate Concentration: Immobilization by varying sodium alginate concentration (1.5%, 3%, 5%) was used for making beads. The highest activity was observed in 5% sodium alginate concentration. (Graph 4)

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Graph 4: Effect of Sodium Alginate Concentration

Storage Stability: The immobilized beads were stored in refrigerator (10ºC) for 25 days and the activity were checked at the interval of 5 days. Immobilized enzyme exhibited decrease in activity after 5th day (Graph 5)



Graph 5: Effect of storage stability (F – Filtrate, W- Wash solution)

CONCLUSION

Total 30 lipase producing bacterial isolates were isolated on nutrient medium supplemented with 1% tween-80 from various oil contaminated soil sample. Isolate VC 13 shows the highest lipase activity and was identified as *Cytobacillus sp.* Results of optimization study on immobilization parameters suggest maximum lipase activity of the selected organism occurs at 37°C, pH 5.5, 50 mg biomass, 5% sodium alginate concentration, and on the 5th day of storage.

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