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Studies on lipase enzyme production from bacillus subtilis under different culture conditions

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Abstract
Lipases or triacylglycerol acyl ester hydrolases are carboxylesterases that catalyze both hydrolysis and synthesis of esters formed from glycerol. Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential. Currently bacterial lipases are of great demand because of potential industrial applications. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butter fat and cream. In the present study the microorganisms where isolated from different soil samples and screened for lipase production on Tributyrin agar medium. The isolate showing higher production of lipase enzyme was identified using standard microbiological techniques. The microorganism was identified as *Bacillus subtilis*. The optimisation for the isolate for the production of the lipase was carried out. The optimisation was carried out for pH, carbon, nitrogen and metal ion source in the production media along with incubation temperature and period for determining the maximum production of lipase by the isolate. The optimised condition where maximum lipase was produced was found to be with pH 8.0, incubation temperature at 37 ^{0}C, incubation period to be between 24-36 hours. The carbon, nitrogen and metal ion source to be optimal for the production of lipase was found to be with olive oil, peptone + yeast extract combination and sodium chloride respectively.

Keywords: Lipase, Enzyme activity, Lipids, Optimisation, Bacillus *subtilis*.

1. Introduction
Lipases hydrolyse triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. These enzymes are of interest in different disciplines in natural science: medicine, biochemistry, biology, the food industry, and in recent years, organic chemistry. Lipases are produced by all biological system viz animals, plants and microorganisms. In comparison to plant and mammalian lipases, microbial lipase are more suitable for industrial application due to their ease of production, relatively inexpensive by fermentation, wide variety and stability. Lipases are produced by wide variety of microorganism.
Currently bacterial lipases are of great demand because of potential industrial applications. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butter fat and cream [1]. Lipases are also used in detergent industry as additive in washing powder [2], textile industry to increase fabric absorbency [3], for synthesis of biodegradable polymers or compounds [4] and different transesterification reactions [5]. In addition, the enzyme is used as a catalyst for production of different products used in cosmetic industry [6], in pulp and paper industry [7], in synthesis of biodiesel [8], degreasing of leather and in pharmaceutical industry [9].
Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and are non- -specific in their substrate specificity and a few bacterial lipases are thermostable [10]. Among bacteria, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Pseudomonas*, *Staphylococcus* and *Chromobacterium* spp. have been exploited for the production of lipases. But in recent years, *Serratia* sp. has been studied for its ability to produce lipase [11].

2. Materials and Methodology
2.1 Isolation and screening of Microorganisms
The microorganisms were isolated from by serially diluting different soil samples rich in lipid like kitchen waste dump sites and community dumpsites and plating them on Tributyrin

agar media plates. The identification of the organism was carried out using microbiological techniques like morphological and Biochemical characterization as per Bergye's manual of determinative bacteriology.

2.2 Screening for Lipase Production:
The screening for the production of lipase by the microorganisms isolated was carried out by plating the isolates on Tributyrin agar medium. The plates were then incubated at 37 ^{0}C for 24 to 48 hours and the zone of hydrolysis around the colonies was determined. The isolate showing the maximum zone was selected as the test organism and identified.

2.3 Lipase Production:
The isolate showing the maximum zone of hydrolysis was selected and cultured in the media containing 0.25g of NaCl, 0.25g of $CaCl_2$, 0.25g of yeast extract and 0.25ml of tween 80, pH 8 for the production of lipase enzyme. The broth was then incubated in shaking condition for 24 to 48 hours at 37 ^{0}C. The lipase assay was carried by PNPP method for determining the amount of lipase produced.

2.4 Optimization for lipase production:
Many Factors like pH, temperature, carbon and nitrogen source, metal ions etc, effects the production of lipase by the isolate. The optimisation was carried out by varying the physical and chemical parameters one at a time. The experiments were conducted in 250ml Erlenmeyer flasks containing 100ml of the production medium.

2.5 Optimization of pH:
The optimisation of the pH was carried out by varying the pH of the production media. The media with different pH "between" 5-9 were inoculated with the isolate. The flasks were then incubated at 37 ^{0}C for 24 to 48 hours and the lipase activity was checked.

2.6 Optimization of Temperature:
The production media was prepared and inoculated with the isolate. The media were then incubated at different temperatures for 24-48 hours to check the effect of temperature in the production of lipase by the isolate. The optimisation of temperature was carried out "between" 25 to 65 ^{0}C.

2.7 Optimization of Incubation period
The isolates were inoculated in the production media and incubated at 37 ^{0}C for different time intervals like 24, 48, 72, 86 and 90 hours. The lipase production was determined for all the incubation period.

2.8 Optimization of Carbon Source
The effect of inducers as a source of carbon on lipase production was studied using olive oil, sunflower oil, castor oil, mustered oil, coconut oil and tween 80 which were substituted. The production of lipase was estimated for each of the carbon source individually after 48 hours of incubation.

2.9 Optimization of Nitrogen source
The effect of Nitrogen source on lipase production was studied using peptone, yeast extract, tryptone, urea, soyabean meal and gelatine which were substituted for the nitrogen source. The media with different nitrogen sources were inoculated with the microorganisms and incubated for 24 hours and the productivity of lipase was determined individually.

2.10 Optimization of Metal Ions:
The effect of metal ions on lipase production was studied using Magnesium chloride, Ammonium sulphate, Sodium chloride, Magnesium sulphate, Zinc chloride and Calcium chloride.

2.11 Mass production
The Mass production of the lipase enzyme was carried out in the media containing Sodium chloride 5 g, Calcium chloride 0.05 g, Yeast extract 2.5 g, Peptone 2.5 g, Tween 80 2.5 ml, Olive oil 2.5 ml, Distilled water 1000 ml and the pH was maintained at 8.0 and the incubation was carried out at 37^{0}C for 48hours.

3. Results
3.1 Isolation of Microorganisms
The microorganisms were isolated on nutrient agar media and subcultured on Tributyrin agar, the isolate showing higher zone of inhibition on Tributyrin agar medium was identified as *Bacillus subtilis* based on the morphological and biochemical characterization as per Bergye's Manual of Determinative Bacteriology. The pure culture was maintained on nutrient agar with olive oil (Figure-1).

Fig 1: Bacillus subtilis pure culture isolate

3.2 Optimisation of pH

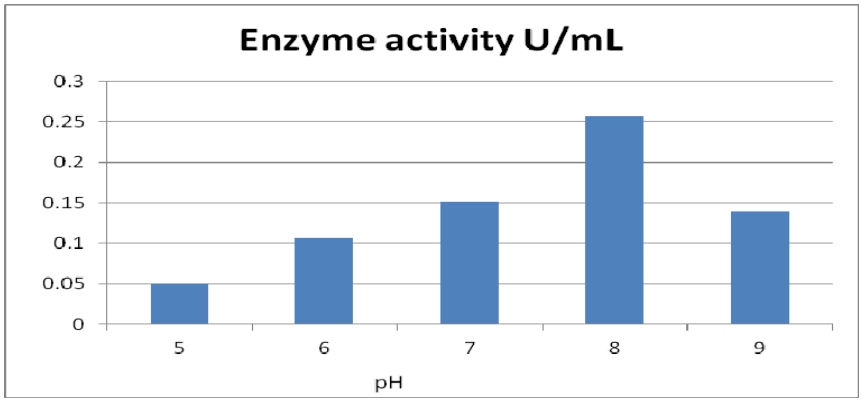

Fig 2: Optimisation of pH.

The optimum pH for maximum lipase production was found to be pH 8.0 (Figure-2), lower pH showed decrease in the amount of lipase enzyme produced.

3.3 Optimisation of Temperature

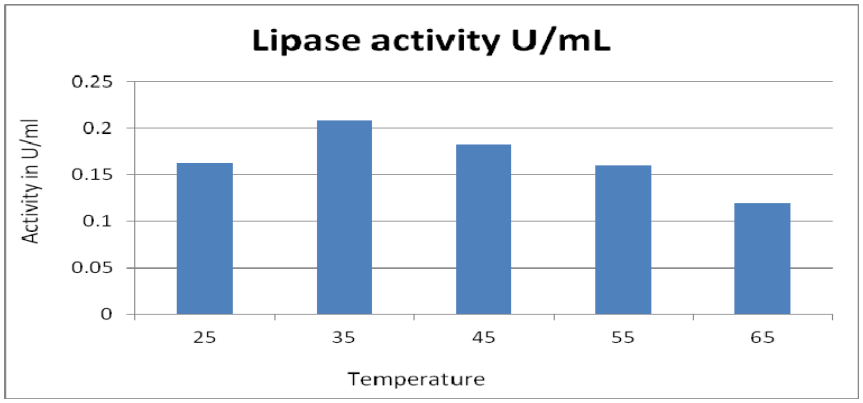

Fig 3: Optimisation of Temperature

The production media inoculated with the microorganism was incubated at different temperature to check the effect of temperature and it was found that the isolate had a higher enzyme activity at 37^0C when compared to other temperatures (Figure-3).

3.4 Optimisation of Incubation period

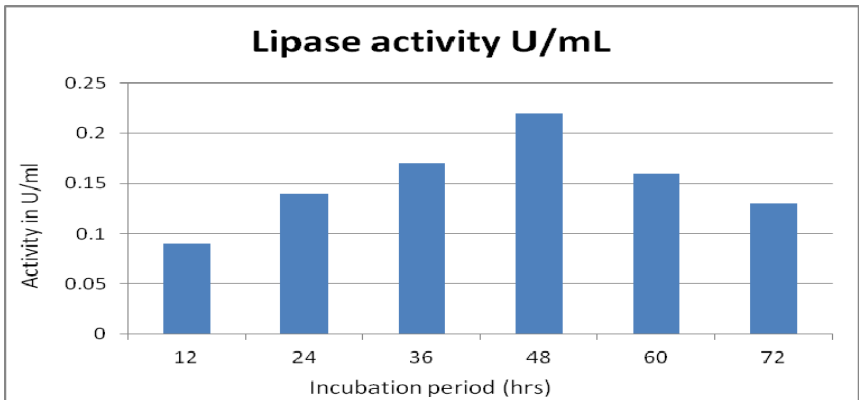

Fig 4: Optimisation of Incubation Period

The incubation period at which maximum lipase enzyme production takes place was carried out by maintaining the

media for different time period. The media was kept at different incubation period ranging from 12-72 hours and the activity was checked at regular interval of time and it was found that the isolate showed a maximum enzyme activity between 36 to 48 hours on incubation (Figure-4).

3.5 Optimisation of Carbon Source

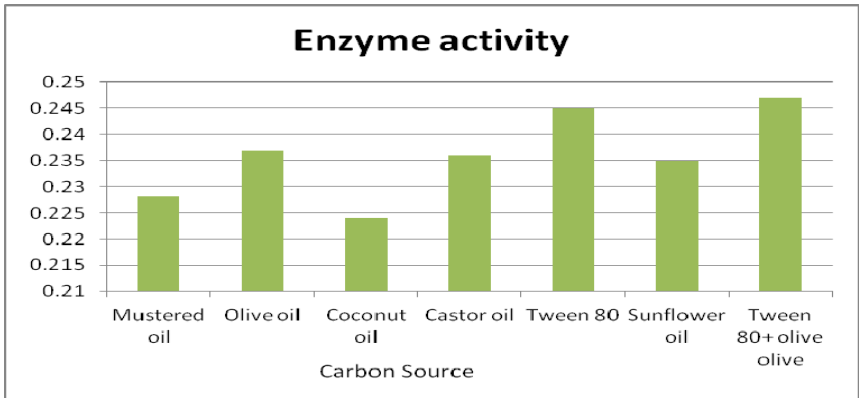

Fig 5: Optimisation of Carbon source

Oils as inducers in the production of lipase enzyme was checked out and was found that the isolate had the ability to produce maximum enzyme in the media containing Tween 80 along with Olive oil (Figure-5).

3.6 Optimisation of Nitrogen source

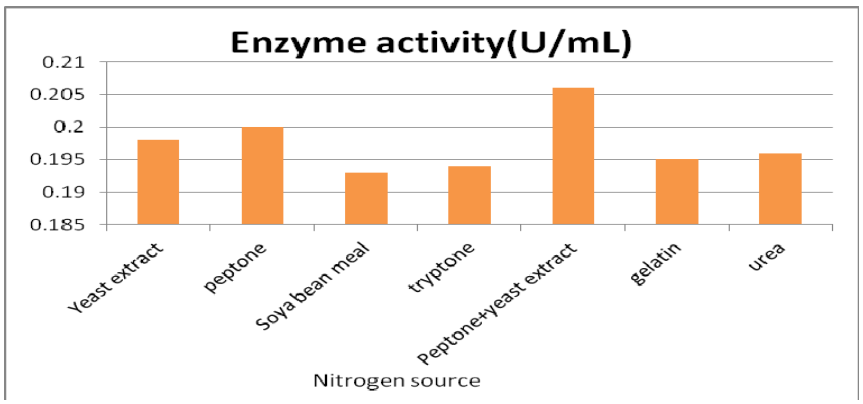

Fig 6: Optimisation of Nitrogen Source

The effect of different Nitrogen sources was checked to analyse the production of lipase by the microorganisms. The production media was substituted with different Nitrogen sources like peptone, yeast extract, tryptone, urea, soyabean meal and gelatine. The activity was checked after 48 hours of incubation and it was found the media containing peptone and yeast extract showed the maximum amount of lipase enzyme (Figure-6).

3.7 Optimisation of Metal Ions

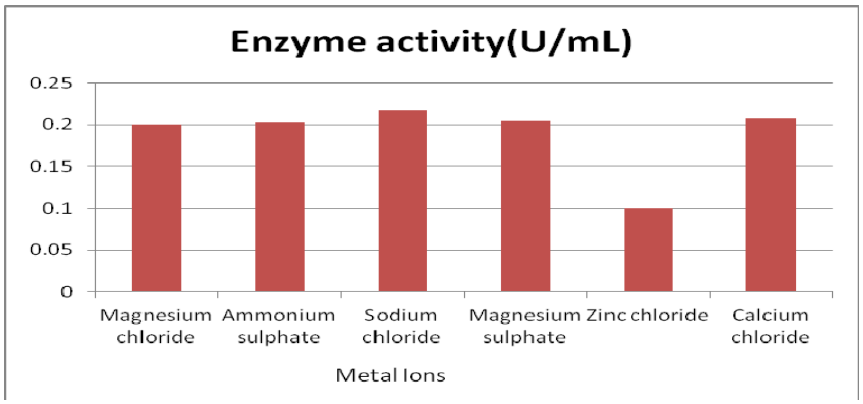

Fig 7: Optimisation of Metal Ions

The optimisation of the metal ion was carried out by substituting the media with different metal ions. The individual media with different metal ions were incubated and the activity for the production of lipase enzyme and it was found that the enzyme produced was maximum in the presence of sodium chloride as a metal ion (Figure-7).

4. Discussion

[12]Investigated the lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. The enzyme activity was very sensitive to the kind and the level of supplementation, and decreased as protease level and pH in the media increased. Maximal levels of glucoamylase and protease were obtained with 4% starch enrichment, indicating that the type of carbon source supplemented to the basal medium determines the major enzymes produced.

[13] Studied lipase production from *Aspergillus niger* by SSF using gingelly oil cake as substrate [14]. Investigated the feasibility of obtaining lipase with *Rhizopus delemar* growing on a polymeric resin [15]. studied the production of lipase by *Aspergillus oryzae* with different solid substrates.

[16], carried out the Investigation of Lipase Production by Milk Isolate *Serratia rubidaea* and observed pH and temperature range optimum for maximum lipase production were 7–8 and 30–40 °C, respectively. With a selected nitrogen source, casein ((6.5±0.015) U/mL) and soytone ((9.4±0.02) U/mL) were suitable substrates for accelerating lipase production.

[17] reported that the lipase production by *Pseudomonas* sp. S34 was maximum with two nitrogen sources, namely trypton and soytone, and the studies by [18] reported that lipase production by *Penicillium aurantiogriseum* was high when using inorganic nitrogen source, but a medium with two organic nitrogen sources displayed lipase production more or less the same to that of the medium containing one organic nitrogen source.

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