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RESEARCH ARTICLE

USE OF SESAME OIL CAKE FOR LIPASE PRODUCTION FROM A NEWLY MARINE ISOLATED *BACILLUS SONORENSIS*

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Abstract

A Gram positive, motile, mesophilic marine *Bacillus sonorensis* strain capable of producing extracellular lipase was isolated from marine clams, *Paphia malabarica*, collected from Kalbhadevi estuary, India. The efficiency of sesame oil cake as a substrate for lipase production by marine *Bacillus sonorensis* strain in submerged fermentation (SmF) has been studied and reported. The sesame oil cake was selected as it is rich in protein, cheap, abundantly available being an agroindustrial waste and can be constructively used as one of the main constituent in medium formulation. Different physical and chemical parameters such as pH, temperature, and substrate concentration and incubation time were optimized. The lipase activity was found to be maximum when pH of medium containing 4% of mustard oil cake was 5.0, at 40°C, after 48 hrs of submerged cultivation. Further, the growth curve studies indicated that the production of extracellular lipase was initiated in the early lag phase and was maximum in the late exponential phase.

Keywords: *Bacillus sonorensis*, marine bacterial strain, lipase, sesame oil cake

Introduction

With the recent advent of biotechnology, there has been an increasing interest and demand for enzymes with novel characteristics. When compared with the terrestrial environment, marine environment gives microorganisms with unique genetic structures and life habitats (Zhang and Kee, 2010). The optimum activity of marine microbial enzymes usually occurs at high salinity, making these enzymes suitable to be used in many harsh industrial processes where the concentrated salt solution used would otherwise inhibit many enzymatic reactions. Most marine microbial enzymes remain stable at room temperature for long periods (Mohapatra *et al.*, 2003). Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) catalyze hydrolysis of triacylglycerides into diacylglycerides, monoacylglycerides, free fatty acids and glycerol at the lipid-water interface. Microbial lipases have been extensively used for biotechnological applications in the food technology, dairy industry, cosmetics, textile and detergent industry (Fariha *et al.*, 2006). In the food industry, lipases play a vital role during the fermentative steps of sausage manufacture and to determine changes in long-chain fatty acid liberated during ripening. Earlier, lipases of different microbial origin have been used for

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refining rice flavour, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine (Seitz, 1974). In addition, they are also known to catalyze other reactions namely esterification, transesterification, inter-esterification between a fatty acid and an alcohol which are the reverse reactions of hydrolysis (Leonov, 2010).

Currently there is a demand on bacterial lipases because of their potential industrial applications. However, their production cost limits their industrial use. Therefore, it is of interest to increase the productivity of fermentation processes by optimization of culture conditions. Since the raw materials employed in the culture medium contribute to total production costs, the reduction in the substrate cost would be a suitable strategy to increase the productivity of the process. Various substrates are used for the production of microbial lipases such as crude coconut fat (Parfene *et al*., 2013), ghee residue (Sahasrabudhe *et al*., 2012), oil cakes etc. India is one of the world's leading oilseeds producing country. Oil cakes have high nutritional value, as they possess high protein content (ranging from 15 to50%). They are economically cheap and abundantly available being an agro-industrial waste product. The potential role of oil cake as a substrate for fermentation has already been established (Ramchandran *et al*., 2007; Babu *et al*., 2011).

The present paper mainly includes screening of lipase producing bacteria from the samples of marine clams, *Paphia malabarica* and evaluation of fermentation process parameters for maximum lipase production from the isolated marine *Bacillus sonorensis* using sesame oil cake as a substrate under submerged fermentation conditions.

Materials and Methods

The chemicals peptone, yeast extract, tributyrin, agar and spirit blue agar were of bacteriological grade and supplied by HiMedia, Mumbai.

Collection and samples treatment

The marine clams (*Paphia malabarica*) were collected from the Kalbadevi estuary, in Mumbai, with the help of local fisherman. In the laboratory, these samples were thoroughly washed with water to remove mud and surface sterilized using 80% ethanol. Under aseptic conditions, the mantle cavity of the clams was opened and the inner soft mass was homogenized with 10 ml of sterile sea water in a mortar and pestle and transferred to a conical flask containing 90 ml of sterile sea water. The flask was shaken for about 5 min at 150 rpm. The flask contents were serially diluted in sterile sea water.

Bacterial isolation, identification and selection

The serially diluted samples were spread on nutrient agar plates and incubated at 40°C for 24-48 hrs and the plates were observed for growth. The 11 bacterial strain isolates so obtained were sub-cultured on nutrient agar plates using the traditional T-shaped streaking method and stored at 4°C till further experiments. All the 11 bacterial strains isolates were screened for lipase production. During screening, a suspension for each of these isolated bacterial strains from the nutrient agar plates was separately made in 0.85% sterile saline and the O.D was adjusted to 0.1.

The preliminary screening was carried out using modified Nutrient Agar. It contained 15g of peptone, 2.5g yeast extract, 0.15g of CaCO3, supplemented with tributyrin [1% v/v], 20g of bacteriological agar made to 1L with seawater. Further pH was adjusted to 7.4 using 1N NaOH. The media was then poured into the Petri dishes and on solidified medium, the prepared serial dilutions were spread using traditional surface spread technique. The plates were incubated at 40°C for 72 hrs and the diameter (D) of the clear hydrolytic zones around the colonies and the diameter (d) of the colonies were measured. The values of ratio D:d were calculated and on basis of this value, the lipolytic strains were selected. The strains so obtained were isolated and maintained on the same modified Nutrient Agar medium and were further screened using spirit blue agar and olive oil.

Broth cultures of the strains obtained by screening with tributyrin were prepared by cultivating the bacterial cultures at 40°C for 48 hrs at pH 7.4 in modified Nutrient Broth which contains all the above mentioned nutrients in same proportion

except Agar. These broth cultures were spread onto the spirit blue agar plates with olive oil emulsion (2% poly vinyl alcohol: olive oil: 3:1) as the substrate. The plates were incubated at 40°C and checked at 24hrs, 48 hrs and 72 hrs for the clearing of deep blue colour around each bacterial colony. Lipase activity was determined by measuring the width of the clear zone around the colony (Parfene *et al.*, 2011; Ranjitha *et al.*, 2009).

The lipase producing strain so obtained was partially characterized by Gram staining, morphological and biochemical studies. The taxonomic identification was carried out at NCCS, University of Pune Campus, using 16s rRNA method.

Selected strain cultivation for lipase production

In the first step, a loopful of freshly grown cells of the selected lipase producing strain were transferred from tributyrin agar plates to a 250 ml of conical flask containing 100 ml of nutrient medium comprising of 3g peptone, 2g yeast extract, 1.5% v/v olive oil, 80% of seawater and incubated at 40°C on a water bath shaker (150 rpm) for 48 hrs. This served as a seed culture for further use as an inoculum. In the second step, 1 ml of the inoculum (4.67 x 10^8CFU/ml) was added to the sterilized medium containing sesame oil cake for fermentation for studying the various optimum growth parameters.

Optimization of the lipase production parameters in submerged fermentation using sesame oil cake

Optimization for the lipase production parameters was carried out through modification of several growth parameters. The effect of an individual parameter was first standardized before standardizing the next parameter. The various parameters optimized for obtaining maximal extracellular lipase production were pH (ranging from 3.0–11.0), temperature (20-80ºC), substrate concentration (1-6%) and incubation time (8-72h) respectively. For studying the optimum incubation time, the extracellular lipase activity was measured every 8 hrs to determine the maximum lipase producing period. For each step lipase activity was assayed to know the optimal yield.

Enzyme Activity Assay

After fermentation, the broth contents were centrifuged at 6000 rpm at 4°C for 20 mins and the supernatent was collected. This supernatant was used as crude lipase sample. The lipase activity was assayed using the quantitative titration method as described by Watanbe *et al.* (1977) with Polyvinyl alcohol and Olive oil (3:1) emulsion as the enzyme substrate. Enzyme activity is expressed as U/ml and one unit of activity is defined as µmols of free fatty acids liberated per ml of crude lipase extract per min under assay conditions.

Statistical Analysis

All determinations were obtained from triplicate measurements and results were expressed as mean ± standard deviation. Student's T test was used to analyse data and statistical significance was declared at $p< 0.05$.

Results and Discussions

Screening of bacteria producing extracellular lipase and identification

Microbiological analysis of the *Paphia malabarica* samples showed around 11 types of bacterial strains isolates. From these 11 bacterial strains, clear hydrolytic zones around colonies were observed in 4 of them on screening with tributyrin. Clear zones around the bacterial colonies indicate the hydrolysis activity of lipolytic enzyme on tributyrin substrate. The formation of zone was observed for every 24 hrs and increased with increase in incubation time. After 72 hrs, however, there was no increase in the size of hydrolysis zone. Then the diameter (D) of the clear hydrolytic zones around the colonies and the diameter (d) of the colonies were measured. The values of ratio D:d were calculated and those colonies having ratio equal to or greater than 1.2 were selected for further screening. Similar screening method was used by Colen et al. (2006) in order to have lipase production. Table 1 shows the values for ratio D/d. The four bacterial strains so obtained were coded as CL1, CL2, CL3 and CL4 with CL1 giving highest D:d ratio value i.e. 2.125. Tributyrin consists of short chain fatty acid, butyric acid (C4). It is generally used for screening of lipase and estimation of lipase activity by titration.

Table 1. *Screening of lipase producing bacteria by stationary cultivation on tributyrin agar medium*

Strain code	Diameter of the clear hydrolytic zone on tributyrin agar medium, mm[D]	Diameter of the bacterial colony on tributyrin agar medium, mm [d]	Values D:d
CL1	8.5	4	2.125
CL2	5.1	3	1.7
CL3	5	4	1.25
CL4	6.4	5	1.28

Table 2. *Screening of lipase producing bacteria by stationary cultivation on olive oil and spirit blue agar medium*

Strain code	Diameter of the clear hydrolytic zone on olive oil + spirit blue agar medium, mm[D]	Diameter of the bacterial colony on olive oil + spirit blue agar medium, mm [d]	Values D:d
CL1	6.5	4	1.6
CL2	3.5	3	1.16
CL3	-	4	-
CL4	-	5	-

However, the clear hydrolytic zone around the colonies may also be due to another enzyme that belongs to the class of lipolytic enzymes that can hydrolyse tributyrin. This other enzyme is esterase (Brockerhoff and Jenson, 1974). Hence, secondary screening of the 4 bacterial strains obtained was carried out using longer chain fatty acids i.e. olive oil in spirit blue agar. This agar is used to identify organisms that are capable of producing the enzyme lipase. Spirit blue agar contains an emulsion of olive oil and spirit blue dye (Starr, 1941). Bacteria that produce lipase will hydrolyze the olive oil and produce a halo zone around the bacterial growth. This enzyme is secreted and hydrolyzes triglycerides to glycerol and three long chain fatty acids. These compounds are small enough to pass through the bacterial cell wall. In spirit blue agar, after 24 hrs, a slight discoloration of deep blue agar was observed in CL1 and CL2 strains. On completion of 48 hrs, the size of the clear hydrolytic zone increased around CL1 colony whereas no change was observed in CL2, CL3 and CL4 strains. The diameter of the hydrolytic zone around CL1 colony was measured and was found to be 6.5 mm.

Table 2 reveals the values of D:d for microbial strains coded as CL1 and CL2 when grown on olive oil and spirit blue agar medium. Hence, it was concluded that the enzyme secreted by CL1 strain is lipase. Thus, from the 4 bacterial strains obtained CL1 was selected for lipase production due to its good lipolytic activity.

Identification of Strain

Gram staining and morphological examinations of CL1 bacterial strain revealed that it is a Gram positive bacteria with rod shaped cells, motile, spore producing and showed pale yellowish-cream colonies on glycerol and tributyrin medium agar plates.

The cells of this strain usually occur singly but few were also observed occurring as colonies of two to four. Palmisano *et al.*, (2001) also observed the same characteristics for *Bacillus sonorensis* isolated from Sonoran desert. The taxonomic identification carried out at NCCS, Pune using 16s rRNA method confirmed that the CL1 strain belongs to the species of *Bacillus sonorensis*.

Effect of pH on lipase production

The pH of the fermentation medium greatly affected the extracellular lipase production of *Bacillus sonorensis* selected strain.

Fig. 1 clearly shows that the extracellular lipase production was maximum in acidic conditions at pH 5.0. Similar results were reported by Guttara *et al.* (2008) where lipase production from *Penicilllium simplicissimum* was maximum in acidic conditions using babassu oil cake as a substrate.

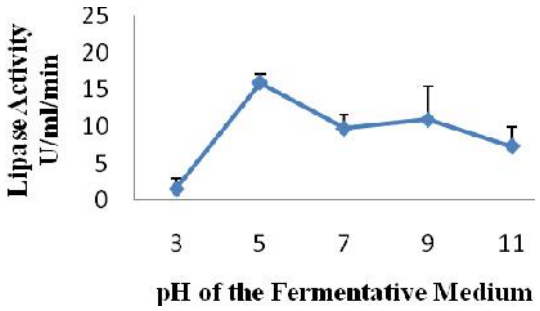

Figure 1 *Effect of pH of the fermentative medium on extracellular lipase production in submerged cultivation system by selected Bacillus sonorensis strain*

Effect of temperature on lipase production

Temperature is one of the major the factors that strongly influences the activity of any microbial enzymes. Microorganisms are very sensitive to temperature changes (Moreira *et al.*, 2002). In the present study maximum extracellular lipase production was observed at 40ºC and till 60ºC it was more or less constant (Fig.2).

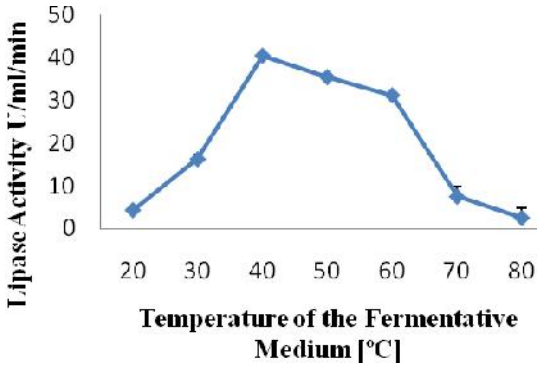

Figure 2 *Effect of temperature of the fermentative medium on extracellular lipase production in submerged cultivation system by selected Bacillus sonorensis strain*

However, beyond 60ºC the lipase activity drastically dropped down indicating that higher temperature is not suitable for lipase production from *Bacillus sonorensis.*

This optimum temperature study is comparable with the study carried out by Gunalaksmi *et al.* (2008) while working on lipase production from *Streptomyces spp.* where lipase activity was maximum at 55ºC and beyond 60ºC, it suddenly dropped down.

Thus, the optimum temperature for extracellular lipase production from *Bacillus sonorensis* selected strain was 40ºC indicating that the strain is a mesophilic microorganism and the further experiments were carried out at this temperature.

Optimum Substrate concentration for lipase Production

Oil cakes have been widely used as substrates for the production of industrial enzymes using fermentation process since they provide both carbon and nitrogen sources in the nutrient medium (Ramchandran *et al.*, 2007). Lipase production from marine *Bacillus licheniformis* using peanut oil cake (Annamalai *et al.*, 2011) and from *Bacillus megaterium* using cotton and neem oil cakes (Sekhon *et. al.*, 2006) under submerged fermentation has been studied and reported. Kuo (1967) has studied and reported the chemical composition of sesame oil cake as follows: 35.6% crude protein, 7.6% crude fibre, 11.8% ash, 2.45% calcium, 1.11% phosphorous.

Thus, it was used as a substrate for lipase production from marine *Bacillus sonorensis*. Fig.3 clearly shows that *Bacillus sonorensis* selected strain exhibits maximum lipase activity in 4% concentration of sesame oil cake in the fermentation medium. Further increase in the concentration inhibited lipase activity.

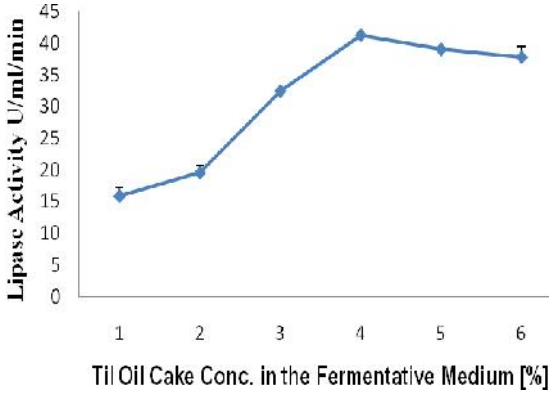

Figure 3 *Effect of concentration of the substrate in the fermentative medium on extracellular lipase production in submerged cultivation system by selected Bacillus sonorensis strain*

Effect of incubation time on extracellular lipase production

Maximum lipase production and bacterial growth was obtained after 48 hrs of incubation time. Fig. 4 reveals that extracellular lipase production from marine *Bacillus sonorensis* selected strain was initiated in the lag phase and was maximum in the late exponential phase. Similar results for lipase production from *Pseudomonas spp.* using groundnut oil cake were also achieved after 48 hrs of incubation by Pannerselvam B. (Pannerselvam *et al.*, 2012). Beyond 48 hrs both the lipase activity as well as the bacterial growth remained more or less constant. *Bacillus spp.* generally synthesize a variety of extracellular hydrolytic enzymes (e.g. amylase, proteases and lipases), the maximum synthesis of which normally occurs in the early stationary and late exponential phases of growth, before sporulation (Priest *et. al.*, 1977). The maximum crude lipase activity after optimizing all production parameters was recorded as 41.35 U/ml/min.

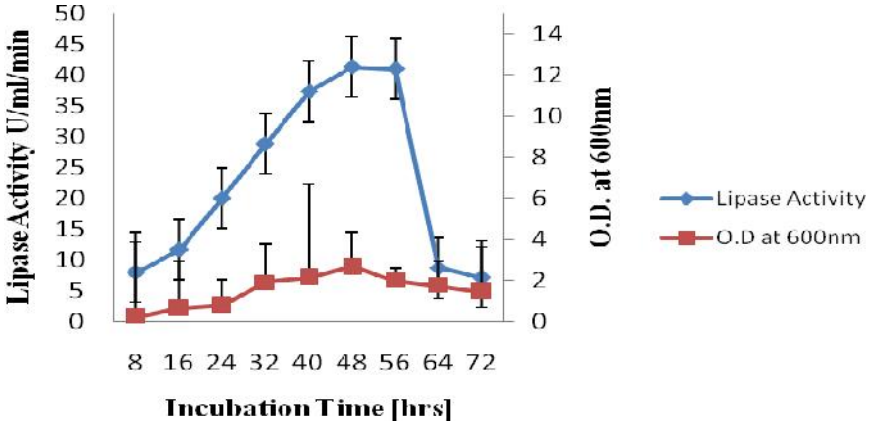

Figure 4 *Effect of incubation time on bacterial growth and extracellular lipase production in submerged cultivation system by selected Bacillus sonorensis strain*

Conclusion

A Gram positive, spore producing marine *Bacillus sonorensis* strain was isolated from marine clams *Paphia malabarica.* The marine *Bacillus sonorensis* strain is capable of producing extracellular lipase. Lipase could be economically produced from *Bacillus sonorensis* using mustard oil cake and seasame oil cakes in submerged fermentation. The fermentation parameters were optimized indicating that the extracellular lipase production from marine *Bacillus sonorensis* strain is maximum in acidic conditions at pH 5.0 under mesophilic conditions at 40ºC during the late exponential growth phase of the bacterial life cycle.

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