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Lipase Production by *Bacillus subtilis* OCR-4 in Solid State Fermentation Using Ground Nut Oil Cakes as Substrate

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Abstract: A moderately alkallophillic strain of *Bacillus subtilis* was isolated and comparative study on the production of extra cellular lipase by Solid State Fermentation (SSF) with various substrates has been evaluated. Different intends such as temperature, pH, different substrates and effect of incubation timeof the medium were optimized for maximum yield. The maximum extracellular lipase activity of4.5 units per gram of dry fermented substrates (Ug/ds) was observed with ground nut oil cake after 48 h of fermentation with 70% initial moisture content of the substrate and suitable growth of bacterial mass culture was for maximum yield of lipase at pH 8 was observed. Solid-state fermentation is a high recovery method for the production of industrial enzymes.

Key words: Lipase, *Bacillus subtilis*, Solid State Fermentation (SSF), substrates

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long chain fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface (Maritinelle *et al*., 1995). Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Only about 2% of world's microorganisms have been tested as enzyme sources. Microbial lipases are produced mostly by submerged culture (Ito *et al*., 2001), but solid-state fermentation methods can be also used (Chisti, 1999). In general, solid-state fermentation is a well-adapted and cheaper process than submerged fermentation for the production of a wide spectrum of bioproducts (animal feed, enzymes, organic acids, biopulp, aroma compounds, antibiotics, compost, biopesticide, biofertilizer etc). Solid-state fermentation is a high recovery method for the production of industrial enzymes (Pandey *et al*., 1999).

It has been reported that in many bioproductions, the amounts of products obtained by solid-state fermentation are many fold higher than those obtained in submerged cultivations. In addition, the products obtained have slightly different properties (e.g., more thermotolerance) when produced in solid-state fermentation and submerged fermentation. Therefore, if solid-state fermentation variables are well controlled and the purity of the product is defined, this technology may be a more competitive process than is commonly thought. Solid-state fermentation offers many advantages over submerged fermentation for production of the enzyme lipase. Coconut cake has been used as a potent substrate for production of lipase by Candida rugosa in solid-state fermentation (Benjamin and Pandey, 1997). High lipase productions were obtained by cultivation of *Rhizopus* sp. (Christen *et al*., 1995; Ul-Haq *et al*., 2002), *Aspergillus* sp. (Kamini *et al*., 1998; Mahadik *et al*., 2002). Recently cheap agricultural by products which have been gaining a great interest as suitable substrates in solid state fermentation for fungi like gingelly oil cake (Kamini *et al*., 1998) and olive oil cake (Cordova *et al*., 1998; Kademi *et al*., 2003). Previous reports had reported that selection of mold strains geared to produce enzymes on solid substrates (Viniegra-González, 1998). Enzyme production can be enhanced by improving the availability of the substrates where Gum Arabic used to emulsify lipid substrates (Mahler *et al*., 2000). Coconut oil cake has been used as a potential raw material for the production of amylase (Ramachandran *et al*., 2004). Olive oil is the most used lipid substrate to induce lipase production by bacteria (Rathi *et al*., 2002). Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and the dissolved oxygen concentration (Elibol and Ozer, 2001). Tropical marine yeast used in mill effluent treatment where palm oil used as substrate by (Oswal *et al*., 2002). Candida rugosa enhanced lipase production in mixed solid substrate fermentation (Benjamin and Pandey, 1998).

Therefore, some important problems associated to solid-state fermentation: designs for up scaling, and control of operations (mainly heat transfer and cooling) and fermentation variables (mainly pH and temperature). Oxygen transfer plays an important role on production of

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lipase (Chen *et al*., 1999). In addition, the diffusion of products through the solid media leads to both extraction processes and purification steps. This contributes to an increase in recovery costs. The present research was undertaken to optimize process condition for the production lipase by solid-state fermentation using oil cakes.

MATERIALS AND METHODS

Sample collection and processing: Soil sample were collected at 4-5 cm depth with the help of sterile spatula in a sterile plastic bag from different petrol bunks in the vicinity of VIT University, Vellore. After collection, sample were brought to the laboratory and 1 g of sample was suspended in 100 mL of sterile distilled water, agitated for 30 min on a shaker at 50ºC and kept as stock solution for further isolation of the microorganism.

Screening of microorganism: Enriched sample was used for plating to get only lipolytic isolates thus enriched samples was plated containing (gm/L): beef extract 3.0, peptone 5.0, sodium chloride 5.0, agar 15.0, calcium chloride 0.05 and glycerol tributyrate 0.2 mL, after incubation for 24 h a total of 8 colonies showing clear zone were picked. One isolate OCR-4, which showed maximum activity was selected and maintained on tributyrin agar slant at 4ºC. The culture was examined for various morphological and biochemical characteristics as per Bergey's Manual of determinative Bacteriology.

Substrate selection: Coconut oil cake, groundnut oil cake, neem oil cake, mustard oil cake, linseed oil cake were used as substrates. Different oil cakes used as substrate and their biotechnological applications (Ramachandran *et al*., 2006). They were procured from a local market of Vellore in March 2008, India and were dried at room temperature to reduce the moisture content and ground to the desired size.

Inoculum preparation: In order to prepare the inoculum, a loopful of cells from a freshly grown slant was transferred into a 250 mL conical flask containing 50 ml of minimal media (without agar) KH_2PO_4 3.0 g, Na_2HPO_4 6.0 g, NaCl 5.0 g, NH_4Cl 2.0 g, $MgSO_4$ 0.1 g in 1 L of distilled water and incubated at 30ºC in a shaking incubator at 180 rpm for 24 h (Oswal *et al*., 2002).

Media preparation: 10 g of desired oil cake was suspended in 90 mL of minimal media in a 250 mL flask. It was then autoclaved at 15 lbs pressure, 120ºC for 20 min. It was cooled before using.

Solid-state fermentation: The above-prepared medium was inoculated with 5 mL of inoculum. After thorough mixing, all the flasks were incubated at desired temperature in a shaking incubator for 48 h. After a stipulated period samples were drawn. The fermented matter was homogenized and a small amount of sample was taken from each flask for extraction and subsequent analysis

Enzyme extraction: The crude enzyme from the fermented material was extracted by simple extraction method. The fermented substrate was mixed thoroughly with 90 mL of 0.05 M of Sorenson phosphate buffer (pH 8.0) and then shaking the mixture in a rotary shaker (180 rpm) at 30ºC for 48 h. The crude enzyme obtained from centrifugation and was used to determine enzyme activity.

Lipase assay: The crude enzyme obtained from centrifugation was assayed for lipase activity. The activity of lipase was determined as described in literature (Winkler and Stuckman, 1979) with the following modification, 10 ml of isopropanol containing 30 mg of p-nitro phenyl acetate was mixed with 90 mL of 0.05 M of Sorenson phosphate buffer (pH 8.0), containing 207.0 mg of sodium deoxycholate and 100 mg of Gum acacia. According to this method a 2.4 mL of freshly prepared p-nitro phenyl acetate substrate solution was mixed with 0.1 mL of crude enzyme. After 15 min of incubation at 15ºC, optical density was measured at 410 nm against an enzyme free control. One unit of lipase activity is defined as the amount of enzyme releasing 1 mole p-nitro phenol per minute under assay conditions.

Optimization of medium parameters: The different parameters selected and optimized (Pau and Omar, 2004) were substrate selection, pH of the medium, incubation time and effect of moisture content of substrate.

RESULTS AND DISCUSSION

Sample collection and screening of microorganism: After incubation of the culture plates, colonies were counted and the bacterial count varied from $1.0x10^8$ to $5.2x10^8$ CFU/g of the soil sample. The serially diluted soil samples were plated on Tributyrin agar and lipolytic count varied from $1.1x10^8$ to $4.9x10^8$ CFU/g of the soil sample. It was found that the soil samples collected from oil refineries waste contaminated sites showed high bacterial count.

Screening of potential bacterial strain for lipase production: On the basis of larger clear zone formation on Tributyrin agar, 08 potential isolates were selected in the study. The isolates were grown in Tributyrin broth at pH 8 and the supernatant were assayed for lipase activity after incubating for 48 h. It was found that the isolate

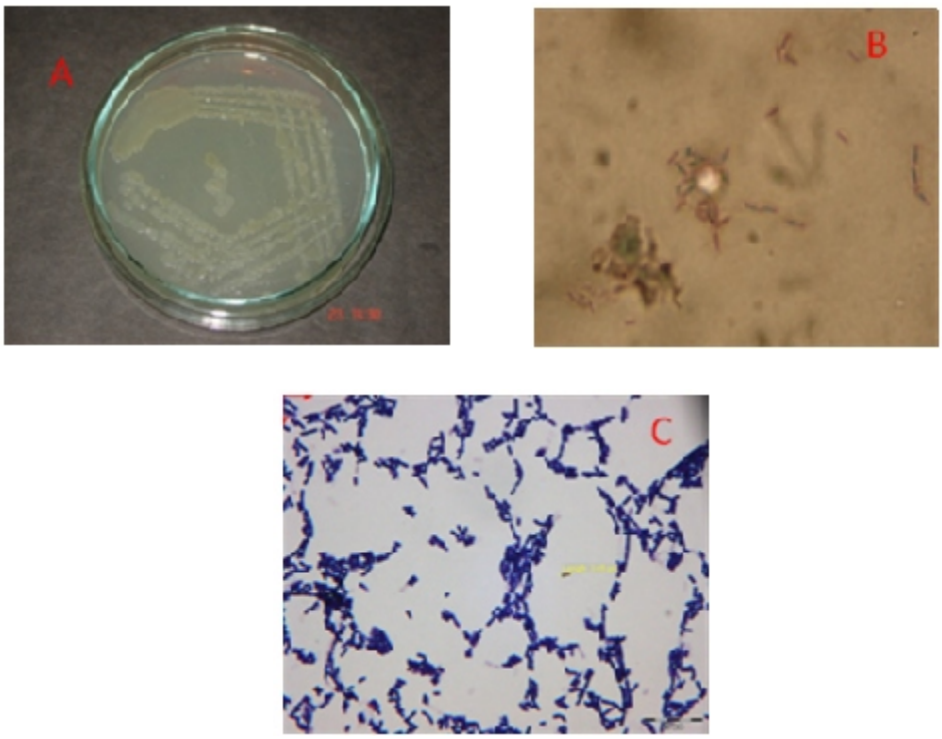

Fig. 1: (A) Oil degrading bacterial stains- *Bacillus subtilis* (B) Endospore staining: Microscopic views at 1000X (C) Microscopic views at 1000X after grams staining showing the dimensions of 3.45 μm

OCR - 04 was found to have highest enzyme production as compared to other isolates. The isolate OCR - 04 was taken for further studies.

Identification of potential strain: The isolate studied was Gram positive, strictly aerobic, rod shaped organism. The organisms were motile. The cells possessed endospore at the centre. The isolate were able to grow at 15 to 42ºC and at wide range of pH 6.5 to 12. On solid media the colonies were round, wavy, convex, rough and opaque. The bacterial isolates showed positive for Voges Proskauer test, Citrate utilization, Casein hydrolysis, Starch hydrolysis, Nitrate and Nitrite reduction, Catalase, Dextrose, Fructose, Lactose and Sucrose.The following characteristics was negative for the strain - growth on MacConkey agar, Indole test, Methyl Red, Gas production from glucose, Urea hydrolysis, H_2S production, Oxidase test. Thus based on biochemical, cultural, and morphological characteristics the isolate were tentatively identified as *Bacillus subtilis* (Fig. 1).

Substrate selection: Among all the substrate, the maximum lipase activity was observed with groundnut oil cake (Table 1). These results were in accordance with observed lipase production from different literature. Different substrate occupied surface area according to their sizes was an important parameter in solid-state fermentation.10 gram of substrate yields maximum production of lipase. Due to its easy penetration, the microbial mass of the bacterial culture showed high growth rate with Groundnut oil cake as a substrate due to which more lipase production was observed. The less lipase production at higher level was due to low mass transfer rate and difficulty in penetration of the organism (Rao *et al*., 2003).

Effect of incubation time: The amount of lipase produced was observed after every 12 h till 60 h. The maximum lipase activity was observed after 48 h of fermentation listed in Table 2. After that, although the bacterial growth rate went on increasing but the specific growth rate decreased. After 48 h, the growth showed divergence from the exponential because in place of homogeneous growth, bacterial pellets began to form in which nutrients and oxygen supply became the growth limiting. After that lipase yield got reduced due to the consumption of nutrient materials.

Effect of initial moisture content of substrate: Variation in initial moisture content of substrate showed

Table 1: Effect of different substrates on lipase activity

Substrates	Lipase activity (U g/ds)
Coconut oil cake	2.35
Groundnut oil cake	4.50
Neem oil cake	1.33
Mustard oil cake	1.68
Linseed oil cake	1.24

Table 2: Effect of incubation time on lipase activity.

Incubation time (h)	Lipase activity (U g/ds)
12	0.30
24	1.65
36	2.00
48	4.50
60	2.81

Table 3: Effect of initial moisture content on lipase activity.

Moisture content % (v/w)	Lipase activity (U g/ds)
30	1.24
40	1.30
50	1.63
60	2.84
70	4.50
80	4.10
90	3.65
100	3.48

Table 4: Effect of pH on lipase activity

pH of the medium	Lipase activity (U g/ds)
6	2.35
7	3.15
8	4.50
9	3.68
10	3.25

that the enzyme synthesis was related to the availability of moisture. Substrate moisture is a crucial factor in SSF and its importance for enzyme production has been well established. With the initial moisture content of 30%, lipase yield was 1.24 U g/ds, which considerably increased with increase in moisture content. The maximum yield was at 70% (4.50 U g/ds) listed in Table 3. Higher moisture would lead to decrease porosity, promotes development of stickiness and increases the chances of contamination (Lonsane *et al*., 1985).

Effect of pH on enzyme production: As pH is the important parameter required for the growth of bacterial culture in respective media so lipase activity got affected with basic pH, this indicates that suitable pH is responsible for bacterial growth in the media. The data obtained clearly indicates that there is a strong influence of pH on lipase enzyme production. Thus the maximum activity was reported at pH 8 (Table 4).

CONCLUSION

Results presented in the Table 1-4 indicate that the various composition influenced enzyme production by the bacteria, it appears that the nature of the substrate had significantly influenced the impact of initial moisture content and incubation period of overall enzyme yield. The physical nature and water holding capacity are important criteria for a solid substrate for its use in SSF process and the moisture content of the medium is a critical factor that determines the microbial growth and product yield in SSF. Fermentation in shake flask improved the lipase yield with an activity 4.50 U g/ds with in 48 h using groundnut oil cake as substrate by *Bacillus subtilis* with moisture content of 70%. Thus this study has proved that the optimization of growth parameters in a suitable solid-state medium has significant effect on improved production. Solid-state fermentation is novel method for higher production of thermostable enzyme with two peaks achieved by optimizing the pH (Ramesh and Lonsane, 1989). This is one of the prime objectives of industrial microbiology for large-scale production of valuable metabolites, which can be achieved with balanced nutrient supply.

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