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Lipase Production from *Bacillus subtilis* MTCC 6808 by Solid State Fermentation Using Ground Nut Oil Cakes as Substrate

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Abstract: A comparative study on the production of extra cellular lipase by Solid State Fermentation (SSF) using Bacillus subtilis with various substrates has been made. Different parameters such as temperature, pH, different substrates and effect of incubation time of the medium were optimized for maximum yield. The maximum extracellular lipase activity of 4.5 units per gram of dry fermented substrates (U g dS^{-1}) 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.

Key words: Lipase, *Bacillus subtilis*, solid state fermentation, substrates, ground nut, tributyrin agar

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 (Martinelle *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: a potent substrate for production of

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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 like gingelly oil cake (Kamini *et al*., 1998) and olive oil cake (Cordova, 1998; Kademi *et al*., 2003) have been gaining a great interest as suitable substrates in solid state fermentation for fungi. Viniegra-González (1998) stated that selection of mold strains geared to produce enzymes on solid substrates. Mahler *et al*. (2000) stated that Gum Arabic used to emulsify lipid substrates can enhance enzymes production by improving the availability of the substrates. Ramachandran *et al*. (2004) stated that coconut oil cake-a potential raw material for the production of amylase. Rathi *et al*. (2002) observed that olive oil is the most used lipid substrate to induce lipase production by bacteria. Elibol and Ozer (2001) stated that 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. Used palm oil as substrate in mill effluent treatment by tropical marine yeast (Oswal *et al*., 2002). Mixed solid substrate fermentation (Benjamin and Pandey, 1998) a novel process for enhanced lipase production by *Candida rugosa*.

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). Effect of oxygen transfer reported by Chen *et al*. (1999) on lipase production. 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

Substrate

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, India and were dried at room temperature to reduce the moisture content and ground to the desired size.

Microorganism

Bacillus subtilis MTCC 6808 obtained from (IMTECH, Chandigarh, India) was used throughout the study.

Growth Conditions

The culture was maintained on tributyrin agar slants having the composition (g L^{-1}): beef extract 3.0, peptone 5.0, sodium chloride 5.0, agar 15.0, calcium chloride 0.05 and glycerol tributyrate 0.2. The pH of the medium was adjusted to 6.0-10.0 and culture was incubated at 30°C for 48 h. The culture was stored at 4°C after sub culturing once in every week.

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 30°C in a shaking incubator at 180 rpm for 24 h (Oswal *et al*., 2002).

Media Preparation

Ten grams 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, 120°C 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 30°C 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 Stuckmann, 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 15°C, 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

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. Ten grams of substrate yields maximum

Table 1: Effect of different substrates on lipase activity

Substrates	Lipase activity (U g dS^{-1})
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^{-1})
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^{-1})
30	1.24
40	1.30
50	1.63
60	2.84
70	4.50
80	4.10
90	3.65
100	3.48

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 (Rhaghavarao *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 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^{-1} which considerably increased with increase in moisture content. The maximum yield was at 70% (4.50 U g dS^{-1}) 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, at pH 6, lipase activity measured was 2.35 U g dS^{-1}, at pH 7, 3.15 U g dS^{-1}, at pH 8, 4.50 U g dS^{-1}, as the pH of the medium was increased lipase activity also increased but after reaching the basic (pH 9 and 10) there was a sudden decrease in enzyme production 3.68 and 3.25 U g dS^{-1}, respectively, 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).

Table 4: Effect of pH on lipase activity

pH of the medium	Lipase activity (U g dS^{-1})
6	2.35
7	3.15
8	4.50
9	3.68
10	3.25

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^{-1} with in 48 h using ground nut 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 for production of higher titres 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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