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BIOSYNTHESIS OF EXTRACELLULAR LIPASES BY *BACILLUS* SP. (MBLB-3) IN RELATION TO THE NUTRITIONAL CONDITIONS

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Abstract

The present study deals with the microbial biosynthesis of lipases by a strain of *Bacillus* sp. Fifteen strains were isolated from oily products. These strains were screened for the production of lipases by solid state fermentation in 250mL Erlenmeyer flasks. Of all the strains examined, MBLB-3 gave maximum production (20.4 ± 0.2^a U/g) of lipases. Different agricultural by-products such as wheat bran, rice husk, almond meal, cotton seed meal, soybean meal, sunflower meal and mustard meal were used as substrates. Maximum extracellular lipase activity (33.9 ± 0.5^a U/g) was observed when almond meal was used as the substrate and it was moistened with phosphate buffer (pH.8.0). The reaction was carried out by taking bacterial cells as a source of lipases in solid substrate fermentation. 0.5 % of Tween 80 was optimized for the maximum production (42.58 ± 0.8^a U/g) of lipases. The above results revealed that the *Bacillus* lipase can be a good additive to be used in detergents.

Introduction

Enzymes are considered as nature's catalysts. Lipase (triacyl glycerol acyl-hydrolases) catalyses hydrolysis of long chain acyl glycerol at an oil water interface. Lipases are special kind of esterases characterized by unique ability to act upon emulsified substrate and hydrolyse glcerides to fatty acids and glycerol (Gilbert, 1993). Lipases are produced by solid state fermentation (Cordova *et al.,* 1998) and submerged fermentation (Abramic, 1999) but at the beginning of this decade solid state fermentation has received increasing interest. This is partly because of the lower energy requirements and less waste water (Lu *et al.,* 1998). Microbial lipases are mostly extracellular and intracellular enzymes which are produced by various fungi (Khan *et al.,* 1998; Pastou *et al.,* 2000) and bacteria (Ito *et al*., 2001; Kim *et al.,* 2002; Mukhtar and Haq, 2008; Haq *et al.,* 2009; Ramini *et al.,* 2010). Bacteria such as *Pseudomonas, Acinobacter, Bacillus* and *Xanthomonas* are well known used for the production of lipases (Nawani *et al.,* 1998; Shen *et al*., 1999; Annamalai *et al.,* 2011). For the production of lipases synthetic and non-synthetic substrates have been used in solid-state fermentation. Piao *et al*., (1998) reported maximum extracellular lipase production by *Pseudomonas stutzeri* strain by using a mixture of ground soybean (1.5 %), corn steep liquor (3 %), glucose (0.5 %) and olive oil (0.75 %) as a substrate. Elwan *et al*., (1983) reported the maximum production of lipase by *Bacillus circulans* at 40 ^oC and optimum pH value was 7.0. The production of lipase by solid state fermentation was very much sensitive to incubation period. (Saleh & Zahran, 1999) found maximum lipase activity after 72 hours of incubation using *Pseudomonas fluorescens.* Sarkar *et al.* (1992) used different bacterial strains for lipase production. They obtained maximum lipase activity, 72 hours after incubation. The lipase producing strains of *Bacillus cereus* and *Bacillus coagulans* were screened by using agar plates of Tween 20 and Tween 80 (Polyoxyethylene sorbitan mono-oleate), (Shafei & Rezkallah, 1998). The thermophilic bacterial strains were maintained on nutrient agar slants containing 1 % Tween 80 and stored at 40^oC (Sidhu *et al.,* 1998). Extracellular lipase producing *Bacillus* sp. were isolated and screened by using agar plates containing olive oil emulsion (Handelsman & Shoham, 1994). Different kinds and concentrations of carbon and nitrogen sources have significant effect on lipase yield. Tween 80 was used as a principal carbon source for the maximum production of lipase by thermophilic *Bacillus* sp. (Fakhreddine *et al.,* 1998). Mahler *et al*., (2000) reported the effect of lactic acid, oleic acid, gum arabic and their interaction on the production of extracellular bacterial lipase. The yield of extracellular lipase increased 2-5 folds by the addition of gum arabic. The present study was designed to optimize the nutritional conditions for the production of bacterial lipases as the bacterial lipases are the ideal source for utilization in the detergent industry.

Materials and Methods

Isolation and screening of lipase producing micro organisms: The bacterial strains capable of producing lipase was isolated from oily products like bread roasted in oil, pickle, sweets (andrasa) and vermicillis (pheonian) taken from local market. Fifteen gram of oily product was transferred to 250 mL conical flask containing 100 mL of sterilized distilled water. The flask was placed on rotary shaker at 200 rpm for 15 min at room temperature. One mL of the above sample was transferred in 100mL of sterilized distilled water in 250 mL Erlenmeyer flask. Five mL of this sample was given heat shock at 80^oC for 15 min (Dulmage, 1970). 0.5 mL of this sample was inoculated on the nutrient agar medium containing g/L tryptone, 10.0; Yeast Extract, 5.0; NaCl, 5.0; Agar, 20.0; Olive oil, 10.0 at pH 7.0. The petriplates were then placed in the incubator at 40^oC for 24 hrs. Colonies with highest clear zone of hydrolysing lipase on the plate were selected as potential lipase producing strain and identified through morphological, physiological and biochemical characteristics. Cultures were then transferred to slants of nutrient agar medium containing nutrient broth 10.0 g/L and agar 20.0 g/L having pH 7.0 (Buchanan *et al.,* 1974).

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Fermentation technique: Ten gram of substrate was moistened with 10 mL of diluent in 250 mL of conical flask. The flasks were autoclaved at 15 lb/inch^2 pressure for 15 min. The flasks were cooled at room temperature. One ml of inoculum was aseptically transferred to each flask. The flasks were then placed in an incubator at 40 ± 2^{o}C for 72 h. The flasks were run parallel in triplicate (Korn & Fujio, 1997).

Extraction of extracellular enzyme: After 72 h, 100 mL of phosphate buffer (pH 7.0) was added to each flask. The flasks were placed on rotary incubator shaker at 200 rpm for one hour at room temperature. After one hour the ingredients of the flasks were filtered and filtrate was used for the estimation of lipase.

Lipase Assay: Lipase activity in fermented meal was determined titrimatrically on the basis of olive oil hydrolysis by the modified method of (Kundu & Pal, 1970). One mL of culture supernatant was added to assay substrate, containing 10 mL of 10% (v/v) homogenized olive oil in 10% (w/v) gum acacia, 2.0 mL of 0.6% $CaCl_2$ solution and 5 mL of phosphate buffer (pH 7.0). The enzyme substrate mixture was incubated on rotary shaker with 150 rpm at 30^{o}C for one hour. 20 mL of alcohol: acetone (1:1) mixture was added to the reaction mixture. Liberated fatty acids were titrated with 0.1N NaOH using phenolphthalein as an indicator. The end point was pink colour.

Lipase unit: A lipase unit is defined as "The amount of enzyme which releases one micromole fatty acid per minute under specified assay conditions".

Statistical analysis: Statistical analysis of results was done according to (Snedecor & Cochran, 1980). Significance has been presented as Duncan multiple ranges in the form of probability (<p>) values.

Results and Discussion

Screening of organism: Fifteen *Bacillus* strains were screened for the production of extracellular lipase by solid state fermentation, using wheat bran as substrate (Table 1). Five isolates of strains of *Bacillus* sp gave low production of enzyme in the range of 5-10 U/g. Eight isolates gave production in the range of 11-15 U/g, while only two isolates ranging from 16-20 U/g (Table 2). Of all the strains tested, MBLB -3 gave maximum lipase production (20.4 ± 0.2^a U/g). Other strains did not exhibit considerable lipase activity, presumably because the enzyme activity was associated with the cell growth (Handelsman & Shoham, 1994).

Table 1. Screening of *Bacillus* sp. for the production of extracellular lipase.

Sr. No.	No. of isolates (*Bacillus* sp.)	Extracellular lipase activity (U/g)
1.	MBLB-1	6.4 ± 0.01^j
2.	MBLB -2	13.3 ± 0.3^d
3.	MBLB -3	20.4 ± 0.2^a
4.	MBLB -4	15.0 ± 0.4^{cd}
5.	MBLB -5	13.3 ± 0.8^d
6.	MBLB -6	11.7 ± 0.03^f
7.	MBLB -7	16.6 ± 0.06^b
8.	MBLB -8	13.4 ± 0.9^d
9.	MBLB -9	8.6 ± 0.1^h
10.	MBLB -10	10.0 ± 0.2^g
11.	MBLB -11	12.2 ± 0.3^e
12.	MBLB -12	5.5 ± 0.4^k
13.	MBLB -13	7.3 ± 0.5^i
14.	MBLB -14	15.1 ± 0.2^c
15.	MBLB -15	12.4 ± 0.3^e

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by the different differ significantly at p≤0.05.
Temperature = 40°C
Incubation period = 72 h
Substrate used = Wheat Bran

Table 2. Sub-grouping of strains of *Bacillus* sp.

No. of strains	Enzyme activity (U/g)
5	5-10
8	11-15
2	16-20

Strains of *Bacillus* sp. having the maximum lipase activity, was selected for all further investigations.

Selection of substrate: Different agricultural by-products were used as substrate and tested with regard to their effect on the lipase production (Fig. 1). Almond meal gave significantly highest enzyme activity (30.10 ± 0.26^a U/g), as compared to other substrates. Almond meal contained gum, asparagin, sucrose and 20% protein (Wallis, 1985). Thus, it was found to be the best source of carbon and nitrogen. Other substrates may not fulfil the nutritional needs of the organism. Hou & Johnston (1992) also used different agricultural by products such as wheat bran, rice husk etc for the production of extracellular lipase.

Effect of incubation period: Incubation period also affects the lipase production. The maximum production of lipase was obtained (31.9 ± 0.31^a U/g) when flasks were incubated for 72 hours (Fig. 2). After 72 hrs there was gradual decrease in lipase production. It might be due to the exhaustion of nutrients in substrate, which resulted in the inactivation of enzyme. This finding is in accordance with Martinez *et al*., 1993; Sarkar *et al.*, 1992; Korn & Fujio, 1997.

Effect of incubation temperature: Incubation temperature also plays an important role in the metabolic processes of an organism. Increasing temperature increased the rate of all physiological processes but beyond certain limits it started decreasing. A range of 20°C to 45°C was employed in the present study (Fig. 3). Maximum lipase activity (33.6 ± 0.22^a U/g) was achieved at 40°C. Thus the incubation temperature of 40.0 ± 0.12°C was optimum for lipase production by solid-state fermentation. Decrease in lipase production can be associated to either decrease in cell growth or inactive nature of enzyme itself. Shafei & Rezkallah (1998) have reported similar results.

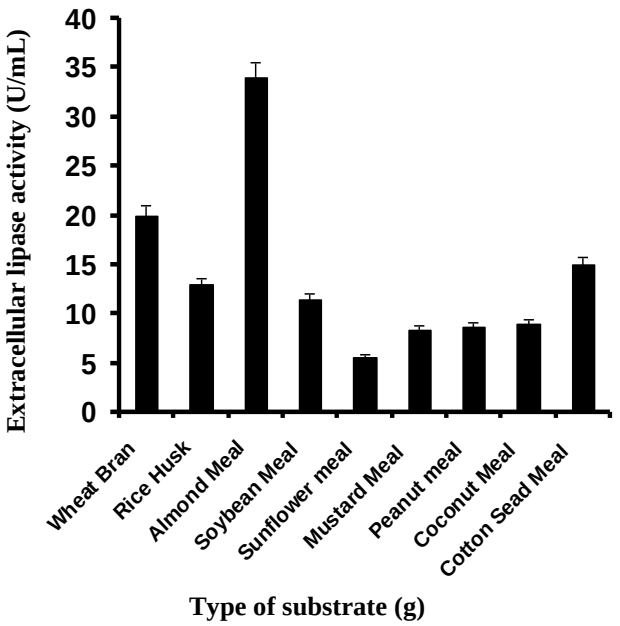

Fig. 1. Selection of Substrate for the production of lipase by strain of *Bacillus* sp.
Each value is an average of three replicates ± denotes standard deviation among replicates.
Temperature = 40ºC
Incubation period = 72 h

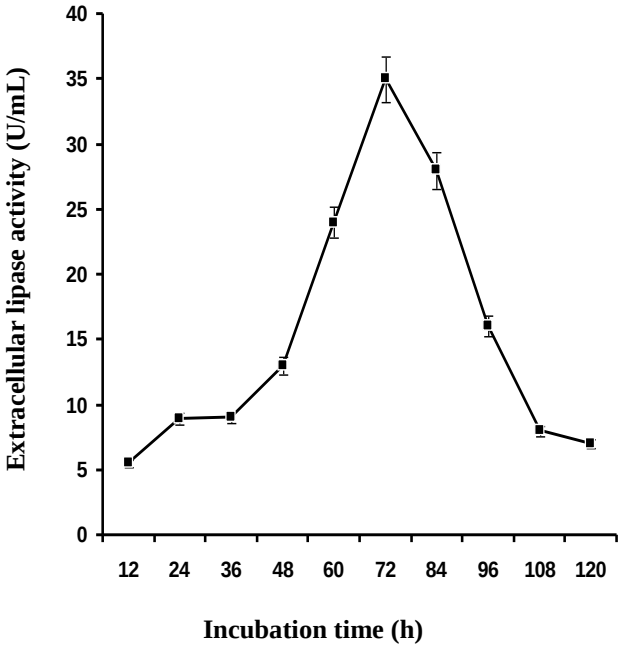

Fig. 2. Effect of incubation period on the production of lipase by strain of *Bacillus* sp.
Each value is an average of three parallel replicates. Y error bar indicate the standard error of mean.
Temperature = 40°C
Substrate used = Almond meal

Effect of inoculum size: The number of cells in inoculum had great influence on the production of lipase by *Bacillus* sp. (Table 3). The size of Inoculum was ranged from 0.5-2.5 mL with an interval of 0.5 mL for the production of lipase by strain of *Bacillus* sp. Highest yield (33.9 ± 0.5[a] U/g) at 1.0 mL of inoculum size, may be due to adequate amount of cells produced, which synthesized optimum level of enzyme. As the number of cells increased, it consumed majority of the substrate for growth purpose, hence enzyme synthesis decreased. Ushio *et al*., 1996 also optimised 1.0 ml of inoculum for maximum lipase production.

Table 3. Effect of size of inoculum on the production of lipase by strain of *Bacillus* sp.

Inoculum size (ml)	Extracellular lipase activity (U/g)
0.5	21.7 ± 0.3[c]
1.0	33.9 ± 0.5[a]
1.5	24.6 ± 0.1[b]
2.0	16.4 ± 0.49 [d]
2.5	9.7 ± 0.21[e]

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
Temperature = 40°C
Incubation period = 72 h
Substrate used = Almond meal.

Effect of different pH of diluent: phosphate buffer has been used as diluent in the present study. pH plays pivotal role in the biosynthesis of an enzyme. A range of pH 4.0 to 8.0 was applied during the experiment. Hence phosphate buffer of pH 8 was optimized for maximum lipase activity as shown in Fig. 4. Same work is also reported by Jaeger *et al*., (1994) and Singh *et al.,* (2010).

Effect of carbon sources: Different Carbon sources such as olive oil, Tween 80, glucose, starch, xylose and sucrose were used as additional carbon sources for the enhancement of lipase activity by strain of *Bacillus* sp. (Fig. 5). 1.0 % additive was added in the fermentation medium and the maximum production of almond meal. Tween 80 gave maximum lipase activity (38.9 ± 0.5[a] U/g). It was miscible with water and did not generally inhibit bacterial growth. Handelsman & Shoham (1994) also reported the production of extracellular lipase by addition of Tween 80 as best carbon source.

Effect of different concentrations of Tween 80: Maximum lipase level (42.58 ± 0.8[a] U/g) was obtained at 1.5 % concentration of Tween 80 (Poly-Oxyethylene sorbitan mono-oleate) as it provided optimum amount of carbon. Enzyme level however decreased with further increase in Tween 80 concentration (Fig. 6). It might be due to fatty acid level accumulating through hydrolysis of substrate, suppressing lipase synthesis. Sidhu *et al.,* 1998 used 0.5 % Tween 80 for the production of extracellular lipase by *Bacillus* sp. Handelsman & Shoham (1994) optimized 1% Tween80 for the maximum lipase production.

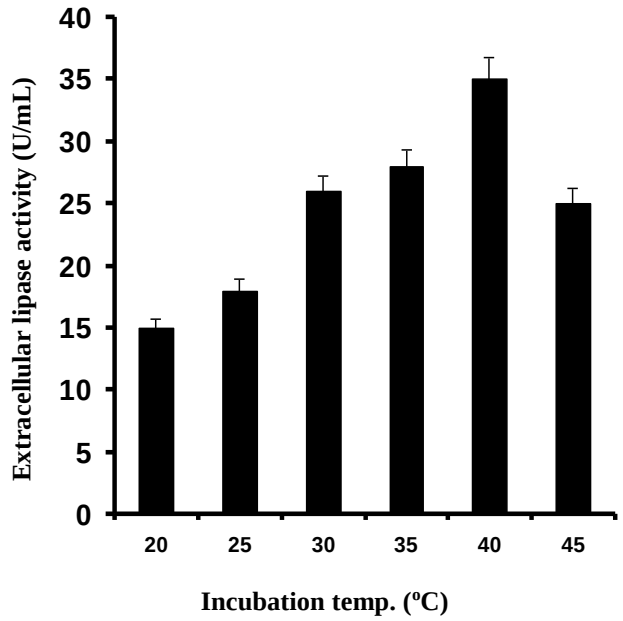

Fig. 3. Effect of incubation temperature on the production of lipase by strain of *Bacillus* sp.
Each value is an average of three parallel replicates. Y error bar indicate the standard error of mean.
Incubation period = 72 h
Substrate used = Almond meal.

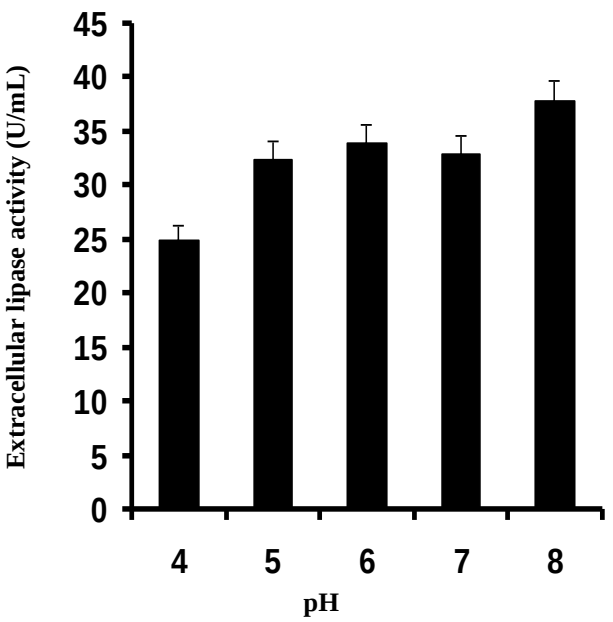

Fig. 4. Effect of different pH of the diluent on the production of lipase by strain of *Bacillus* sp.
Each value is an average of three parallel replicates. Y error bar indicate the standard error of mean.
Incubation period = 72 h
Substrate used = Almond meal.

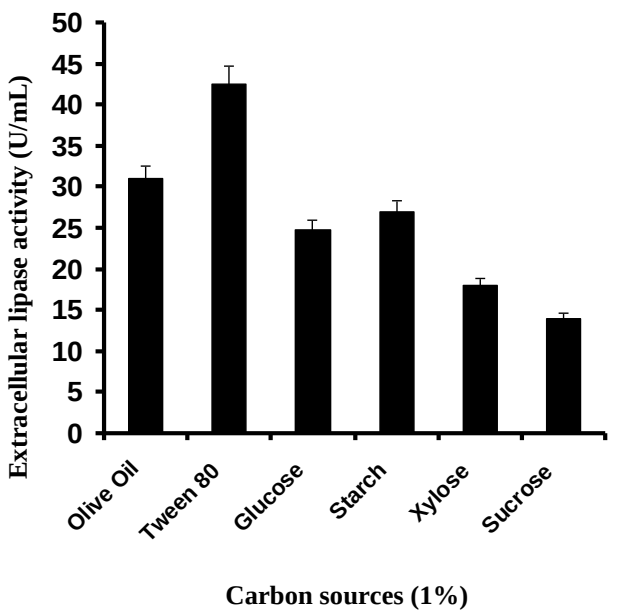

Fig. 5. Effect of Carbon sources on the production of lipase by strain of *Bacillus* sp.
Each value is an average of three replicates ± denotes standard deviation among replicates.
Temperature = 40ºC
Incubation period = 72 h

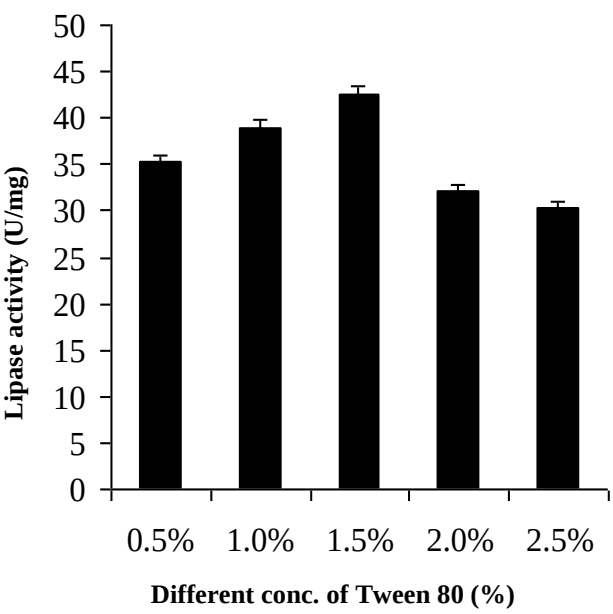

Fig. 6. Effect of Different concentrations of Tween 80 on the production of lipase by strain of *Bacillus* sp.
Each value is an average of three replicates ± denotes standard deviation among replicates.
Temperature = 40ºC
Incubation period = 72 h

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