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Economic production of polyethylene modifying lipase enzyme under solid state fermentation using banana peels and sand

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ABSTRACT

Agro –industrial by-product residues were used as substrates in the presence of sand as a carrier material for production of polyethylene surface modifying lipase enzyme under solid-state fermentation from *Bacillus* isolate W5. Banana peels supported the highest enzyme production at 4.5-6% (732 U/g of fermented culture). Maximum lipase production was achieved at 10% initial moisture content and 30°C incubation temperature. Enrichment of the medium with ammonium acetate as carbon sources increased the enzyme production about 31%. On the other hand, the nitrogen source, peptone enhanced the enzyme production by 28%. Optimum initial pH and incubation period were pH 7 and 5 days, respectively. Calcium chloride and potassium chloride at 1% enhanced enzyme productivity by 12% and 9% respectively. Supplementation the medium with surfactant has no effect on enzyme productivity. Pilot scale production of lipase enzyme under the optimum SSF conditions was tested in aluminum trays. The lipase activity was 1397 U/g fermented culture. This result was comparable with that obtained from bench scale production (in flasks). The cost of one kilogram of this fermented culture containing 1397000 U of lipase was estimated as US $ 2.4. The enzyme showed the highest activity at 70°C and pH 8.5. It was stable up to 50°C for 15 min and pH range 5-9 for one hour.
The obtained results were discussed in the light of possible utilization of banana peels and sand for the production of this important industrial enzyme under solid state fermentation.

KEYWORDS

Polyethylene surface modifying lipase;
Banana peels;
Sand;
Bacillus;
Solid state fermentation.

INTRODUCTION

In the last few years there has been increasing interest in enzymatic surface modification of poly(ethyleneterephthalate) (PET), a synthetic polymer which widely used in the textile industry with an annual production of 36 million tons[4,13]. PET shows excellent properties but in the same time have some undesired properties such as perspiration cannot penetrate the fabrics and lower chemical reactivity due to low

dyeability, difficulties in finishing, build-up of electrostatic charge and the tendency to pilling[27]. Enhancement of the hydrophilicity of PET is a key requirement for many applications and can be achieved by chemical, physical and enzymatic methods. Enzyme treatment can be chosen as a green alternative for synthetic polymer surface modification as they offer many advantages over chemical and physical methods such as they are very specific, act under moderate reaction conditions which lead to less or negligible damage of the strength properties of the synthetic polymers, easier to control, cost effective, eco-friendly and the scale up is possible[5,39]. Due to the size of enzymes and the insoluble nature of PET fiber in an aqueous medium, the enzymes are merely active at the surface so that the bulk characteristics of the fibers remain unchanged.

Enzymes active on PET substrates include various cutinase, lipases and esterases. The enzymatic modification of PET implies the limited hydrolysis of backbone ester bonds, which generates new free hydroxyl and carboxyl groups at the polymer surface, thus leading to increased hydrophilicity of the PET substrate[14,17,31,32,35,40].

A number of studies have been carried out on the surface modification of PET fibers using lipase enzymes[5]. The improvement of wettability, dyeability, high cationic dye binding and oily stain resistance of PET fabrics treated with polyesterase was reported by[40]. The effect of nine commercial lipases on PET fabrics was analyzed by[18] and they reported that moisture regain of PET fibers was improved by 2.4 times as compared to alkaline treatment and also the carboxyl and hydroxyl groups had been successfully introduced. Similarly, lipase from *T. lanuginosus* was used to increase the hydrophilicity of PET fabrics by Bruckner *et al.*, 2008. Owing to an increase in the number of hydroxyl groups, reactive dye showed more intense color, which was confirmed by reflectance spectroscopy and an enhancement in their water-absorption ability[4].

Over the past couple of years, solid-state fermentation (SSF) involving growth of microbes on moist solid substrate(s) in the absence of free flowing water, has gained a tremendous momentum owing to certain advantages over the conventional submerged fermentation, like low production cost, saving of water and energy, less waste effluent problem and stability of the product due to less dilution in the medium[15,23,24,30].

SSF has been used to produce lipases from various by-products of agriculture and industrial origin that have little commercial value[19].

The authors have previously screened 19 lipase enzymes produced by bacilli isolated from Egypt for specific surface modification of PET fabric. One of these enzymes produced by *Bacillus* isolate 5W (identified as *Bacillus subtilis* showed good and promising results in modifying PET surface[1,11].

EXPERIMENTAL

Bacteria used and inoculum preparation

Bacillus isolate 5W was isolated from Giza governorate in Egypt and used in this study for production of PET modifying lipease enzyme[11]. The tested organism was grown in nutrient broth at 30°C for 24 h under shaking at 150 rpm for inoculum preparation.

Agro-industrial by-products used

Agro-industrial by-products were supplied by Oil Extraction Unit and Animal Nutrition Department at National Research Center of Egypt. These agro-industrial by-products included sugar beet pulp, coconut meal, jojoba meal, cress meal, linen meal, wheat germ meal, cotton seed meal, pea peels, potato peels, orange peels, banana peels, bean peels and carrot pomace.

SSF and optimization of the process parameters

A group of dried ground agro-industrial by-products was employed as the main source of nutrients for growth and lipase production by *Bacillus* isolate 5W without any pretreatment in the presence of sand as carrier material.

Initially 50 g of sand and 3% of each substrate were taken individually in 250 ml Erlenmeyer flasks, moistened with tap water at 10% moisture and autoclaved. Inoculum of 14×10^6 CFU/g was added and incubated at 30°C for 5 days under static conditions. Each fermentation test was repeated twice in triplicate.

Effect of banana peels concentrations (1.5 – 9%), moisture content (5-80%), carbon source at 1% (glucose, galactose, arabinose, fructose, raffinose, maltose, sucrose, lactose, starch, cellulose, pectin, ammonium acetate, sodium citrate and glycerol), nitrogen source

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at 1% (sodium nitrite, sodium nitrate, ammonium sulphate, ammonium phosphate, urea, peptone, yeast extract and malt extract), initial pH (5.7-9), incubation temperature (20-50°C), incubation period (3-15 days), metal ions at 1% (Na^+, K^+, Mg^{2+}, Fe^{2+}, Ca^{2+}, Co^{2+} and Mn^{2+}) and surfactants at 0.1% such as Tweens (Tween 20, Tween 40, Tween 60, Tween 80), Triton X-100 and gum arabic were studied.

Also, pilot-scale production of lipase enzyme by the tested organism in aluminum trays ($30\times30\times10$ cm^3) under the optimum SSF conditions was tested. Each tray contains 850 gram sand, 4.5% banana peels, 1% ammonium acetate, 1% peptone, 1% $CaCl_2$ and 10% moisture content was inoculated and incubated for 5 days at 30°C.

Enzyme extraction

The enzyme from the fermented culture was extracted with tap water. The slurry was squeezed through a damp cheese cloth. Extracts were pooled and centrifuged at 4°C for 15 min at 10.000 rpm. The supernatant was used as the enzyme source.

Lipase activity assay

Lipase activity was assayed according to[9,21] with some modifications. Lipase activity was determined using *p*nitrophenyl acetate (*p*-NPA) as a substrate. The substrate solution was prepared by dissolving *p*-NPA in isopropanol. The mixture of 880µl of 50mM phosphate buffer (pH 8), 20µl of substrate solution and 100µl of suitably diluted enzyme solution was incubated at the tested temperature for 30min. The reaction was terminated by the addition of 0.2ml of 100mM $CaCl_2$ solution (at 0°C) and keeping it on ice. The reaction mixture was centrifuged to clarify the solution and the absorbance of the yellow color of the supernatant was read at 410nm.

One enzyme unit was defined as a 0.1 increase in OD_{410} under the standard assay conditions.

Biochemical properties of the enzyme

Temperature and pH profiles were determined at different temperatures (30-70°C) and pH rang 7-9, respectively. The thermal and pH stabilities of the enzyme were ascertained by measuring the residual activity of the enzyme exposed at various temperatures (30-70°C) for 15 minutes and at pH range 7-9 for one h, respectively. Effect of metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}) at 5mM were tested. All experiments were conducted in triplicates.

Statistical analysis

All the experiments were carried out independently in triplicates in 250-ml Erlenmeyer flasks. The data represented here are in the form of mean ± SE. All the values were subjected to one way analysis of variance (ANOVA) and significance is presented as Duncan's multiple range test results in the form of probability ($P \leq 0.05$) values which were obtained using SPSS.

RESULTS AND DISCUSSION

Evaluation of agro-industrial by-products as substrates for lipase production

Lipases are valuable biocatalysts have diverse applications. Though lipase enzymes share only 5 % of the industrial enzyme market, they gained focus as biotechnologically valuable enzymes[18].

The selection of an ideal agro-industrial residue for enzyme production in a SSF process depends upon several factors, mainly related with the cost and avail-

TABLE 1 : Effect of different agro-industrial by-products as substrates on lipase production by tested organism under SSF

Agro-industrial by-products	Lipase activity* (U/g fermented culture)
Sugar beet pulp	270 ± 10 g
Coconut meal	533 ± 7.3 d
Jojoba meal	289 ± 12.3 efg
Cress meal	315 ± 14 ef
Linen meal	318 ± 9.6 e
Sesame meal	303 ± 8.8 efg
Wheat germ meal	322 ± 12.7 e
Cotton seed meal	650 ± 18.7 b
Pea peels	276 ± 6.4 fg
Potato peels	288 ± 13 efg
Orange peels	263 ± 11.7 g
Banana peels	732 ± 10 a
Bean peels	527 ± 15.6 c
Carrot pomace	585 ± 8.7 c

*** Lipase activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at $P \leq 0.05$**

ability of the substrate material, and thus may involve screening of several industrial residues[26].

Among the tested agro-industrial by-products, banana peels yielded the highest lipolytic activity (732 U/g fermented culture) followed by cotton seed meal (650 U/g fermented culture) as shown in TABLE 1. The optimum banana peels concentration for the maximum lipase production was 4.5- 6% as shown in Figure 1. Lower or higher concentrations of banana peels decreased the enzyme productivity.

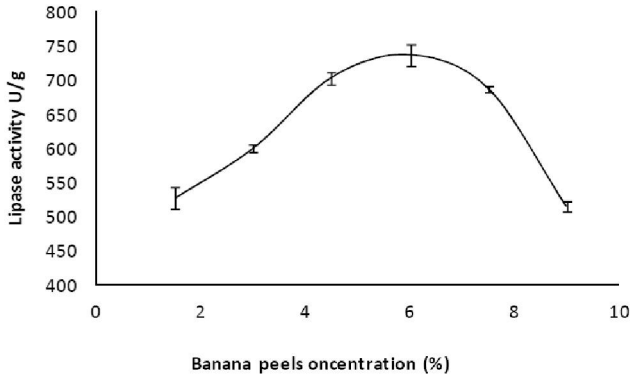

Figure 1 : Effect of banana peels concentration on lipase production by *Bacillus* isolate 5W organism under SSF

The banana yield is over 145 million tonnes in 2011, there is a significant amount of banana peels waste being generated. Banana peels contain 0.9% dry matter of protein, 1.7% crude lipid, 59% carbohydrate, 20-30% fiber, 0.08 % potassium, 0.02% calcium, 0.02% sodium, 0.06% manganese and very low concentrations of other numerous elements (Anhwange *et al.*, 2009, http://www.ehow.com/info_10033568_components-banana-peel.html).

In the literature different natural substrates namely sugar beet pulp, olive oil cake, soy cake, coconut oil cake, babasu cake, gingelly oil cake, rice bran, wheat bran, almond meal, sugar can bagasse, rise husks, melon wastes, ground nut oil cake, mustard oil cake and *Jatropha curcas* used for lipase production under SSF[2,8,16,20,22,29,33,34,36,38].

Using sand in this study perhaps has some benefits as a carrier material in SSF as for instance it does not absorb water, allowing more air to penetrate, occupies a small volume and it can be reused several times.

Influence of moisture content on enzyme production

Among the several factors that are important for microbial growth and enzyme production under SSF using a particular substrate, moisture level which is one of the most critical factors. SSF process is different from submerged fermentation culturing, since microbial growth and product formation occurs at or near the surface particle having low moisture content[26,28].

In the present study, the maximum enzyme production was obtained at 10% moisture content (727 U/g fermented culture) as shown in Figure 2. Further increase in moisture level in the fermentation medium resulted in reduction of lipase production.

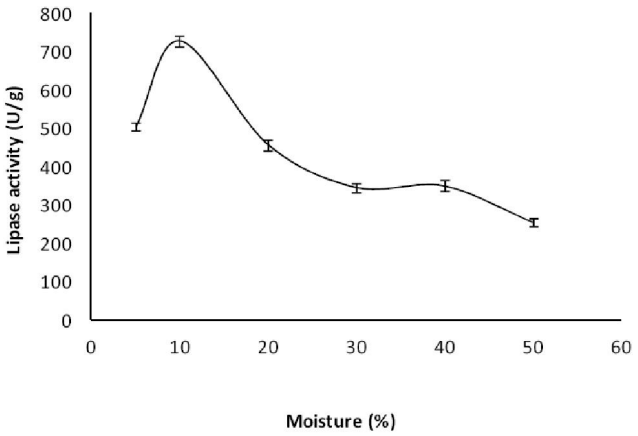

Figure 2 : Effect of moisture content on lipase production by *Bacillus* isolate 5W under SSF

TABLE 2 : Effect of carbon sources on lipase production by tested organism under SSF

Carbon sources	Lipase activity* (U/g fermented culture)
None (control)	713 ± 4.4 e
Glucose	766 ± 14.2 d
Galactose	712 ± 6.4 e
Arabinose	619 ± 11 f
Fructose	765 ± 13 d
Raffinose	873 ± 15 b
Maltose	803 ± 3.7 c
Sucrose	767 ± 12 d
Lactose	786 ±8.4 cd
Starch	581 ±10 g
Cellulose	526 ±9.3 h
Pectin	605 ±7.8 fg
Ammonium acetate	933 ±18.5 a
Sodium citrate	38 ±1.7 i
Glycerol	39 ±2.2 i

*** Lipase activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P ≤ 0.05**

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In the literature, the optimal moisture level for lipase production by bacilli has been reported to be 50% -75%[8,16,20]. Low moisture level used in this study is related to the nature of the carrier material (sand) which has zero water absorbance.

Pandey *et al.*, 2000 reported that lower moisture levels lead to reduced solubility of the nutrients in the solid substrates, a lower degree of substrates swelling and higher water tension. Similarly, higher moisture content were reported to cause decreased porosity, loss of particulate structure, development of stickiness, reduction in gas volume and decreased gas exchange.

Effects of carbon sources on enzyme production

Various tested carbon sources showed different impact on enzyme production as shown in TABLE 2. Ammonium acetate (933 U/g fermented culture) followed by raffinose (873 U/g fermented culture) enhanced the lipase production about 31% and 22%, respectively. On the other hand, arabinose, starch, cellulose, pectin, citrate and glycerol caused varied repression effect on lipase activity.

It was found that maltose and starch were the best carbon sources for production of lipase by *Pseudomonas aeroginosa* PseA [16,20], *Bacillus coagulans*[2] and *Bacillus subtilis* OCR-4[34] in solid state fermentation.

Effect of nitrogen sources on enzyme production

Nitrogen sources have dramatic influence on enzyme production and play a crucial role in enzyme induction in bacteria[12].

Among the various tested nitrogen sources, organic nitrogen sources increased lipase biosynthesis. Thus, peptone followed by yeast extract at 1% was found to be the best nitrogen sources for the enzyme production where they lead to 28 and 23% increase in lipase production, respectively as shown in TABLE 3. On the other hand inorganic nitrogen sources repressed the enzyme production to different levels. These results are in agreement with those reported by[12] 2012 who found that peptone was the best nitrogen source for lipase production by *Bacillus subtilis* OCR-4. On the other hand, Alkan *et al.*[2] and Mahanta *et al.*[20] reported that NH_4NO_3 and $NaNO_3$ respectively were the best nitrogen sources for enzyme production.

TABLE 3 : Effect of nitrogen sources on lipase production by *Bacillus* isolate 5 Wunder SSF

Nitrogen sources	Lipase activity* (U/g fermented culture)
None (control)	933 ± 9.9 d
Sodium nitrite	603 ± 11 g
Sodium nitrate	785 ± 10.5 e
Ammonium sulphate	719 ± 13 f
Urea	578 ± 11.6 g
Peptone	1195 ± 6 a
Yeast extract	1148 ± 14.8 b
Malt extract	1012 ± 19.6 c

*** Lipase activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P ≤ 0.05**

TABLE 4 : Effect of initial pH on lipase production by *Bacillus* isolate 5W under SSF

Initial pH	Lipase activity* (U/g fermented culture)
Control	1182 ± 5 b
5.7	1130 ± 8.7 c
6	1149 ± 17.7 bc
5.5	1153 ± 6 bc
7	1232 ± 19 a
7.5	1140 ± 10.7 c
8	1120 ± 12 c
8.5	885 ± 9 d
9	877 ± 9.3 d

*** Lipase activity is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P ≤ 0.05**

Microbial growth and metabolism inevitably lead to a change in the hydrogen ion balance and hence, the pH of the culture medium. TABLE 4 shows that, as initial pH increased, lipase production increased reaching its maximum level at pH 7 (1232 U/g fermented culture) followed by a reduction in lipase production with increasing initial pH value. These results are in agreement with those results reported by Alkan *et al.*[2], Mahanta *et al.*[20] and Bora and Kalita[6]. In contrast, Chaturvedi *et al*[8] and Immanuel *et al.*[16] reported that pH 8 and pH 9 were the optima for lipase production by their tested organisms, respectively.

Effect of incubation temperature

It is known that the temperature is one of the most critical parameter that has to be controlled in any

bioprocess[10].

Bacillus isolate 5W exhibited the highest enzyme production at 30°C (1228U/g fermented culture) as shown in Figure 3. Increasing the cultivation temperature resulted in reduction of enzyme production. It was reported that the optimum temperatures for lipase production by bacilli were 37°C, 50°C and 60°C as reported by Alkan *et al.*[2]; Bora and Kalita[8] and Immanuel *et al.*[16], respectively.

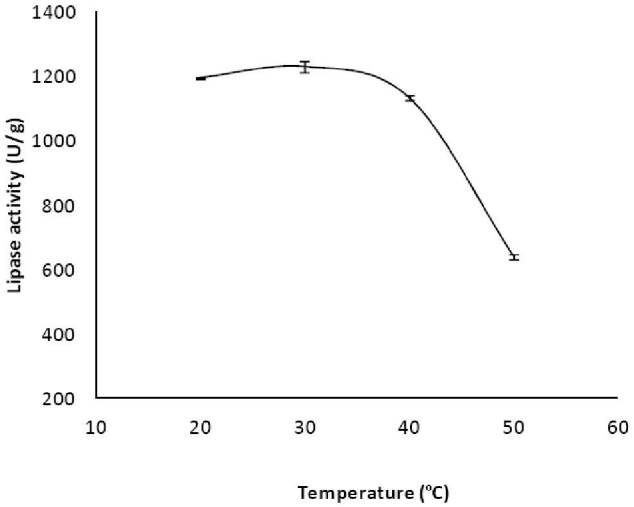

Figure 3 : Effect of incubation temperature on lipase production by *Bacillus* isolate 5W under SSF

Effect of incubation period on enzyme productivity

It is extremely essential to detect the optimum incubation time at which an organism exhibits the highest enzyme activity since organisms show considerable variation at different incubation periods.

The time course study of enzyme production under SSF condition showed an increase in lipase production with optimum incubation time at 5th day (1244 U/g fermented culture) as shown in Figure 4. Further incubation time showed reduction in enzyme activity. The decline in enzyme activity might be due to denaturation and/or decomposition of lipase enzyme as a result of interactions with other compounds in the fermented medium[37]. In most of the reports the maximum lipase production was reported between 24h-192h[2,6,8].

Effect of metal ions

Among the tested metal salts, calcium chloride, potassium chloride, sodium chloride and manganous chloride enhanced the lipase production about 12%, 9%, 6% and 5%, respectively (data not shown). Other metal ions had no effect on enzyme production. Positive effects of metal cations on lipase production have been reported for Ca^{2+}, Na^{+}, K^{+} and Mg^{2+} by Immanuel *et al.*,[16] and Bora and Kalita[6]. However, supplementation of metal ions like Mn^{2+} and Ni^{2+} ions showed repressive effect on lipase production as reported by Alkan *et al.*,[2].

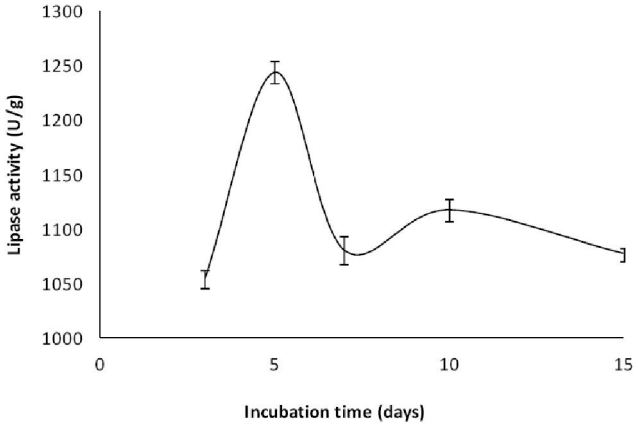

Figure 4 : Effect of incubation period on lipase production by *Bacillus* isolate 5W under SSF

TABLE 5 : Cost of the medium ingredients for production of 1 kg of fermented culture for lipase enzyme production

Contents	Cost ($)
Sand (50 g)	0.00034
Banana peels (3 g)	0.0
Ammonium acetate (0.53g)	0.033
peptone (0.53)	0.102
Tap water (5.3 ml)	0.0000035
Seed culture	0.0063
Total quantity	60.36g
Total price	$ 0.142
One kg product price	$ 2.35
Units/gram	2450

One unit of the enzyme activity was defined as a 0.1 increase in OD_{410} of the reaction mixture. *p*-nitrophenyl acetate (*p*-NPA) was used as a substrate.

Effect of surfactant on enzyme production

Addition of different Tweens (Tween20, Tween40, Tween60 and Tween80), Arabic gum, and Triton X-100 to the medium had no effect on lipase production by *Bacillus* isolate 5W (data not shown).

Pilot-scale production of lipase enzyme under SSF conditions in trays and its cost

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Production of PET surface modifying lipase enzyme in trays ($30 \times 30 \times 10$ cm^3) under the optimum SSF conditions showed lipase activity of 1397 U/g fermented culture.

The ingredients cost for production of one kilogram wet weight of fermented culture in aluminum trays under SSF optimum conditions containing 1397000 U lipase enzyme was estimated as US $ 2.4 (TABLE 5).

Enzyme characterization

The optimum temperature and pH for the highest lipase activity were obtained at 70ºC and pH 8.5, respectively. It was stable at 50ºC for 15 min and in pH rang 7-9 for one h. Addition of metal ions like Mg^{2+}, Ca^{2+} and K^{+} has no effect on the enzyme activity however, 5-10% repression in lipase activity was recorded after addition of Fe^{2+}, Na^{+}, Zn^{2+}, Mn^{2+} and Cu^{2+} ions.

Finally, it is of interest to note that the present work constitutes the cost effective medium for production of PET surface modifying lipase enzyme produced by *Bacillus* isolate 5W on banana peels using sand as carrier under SSF technology. This approach is expected to be highly feasible and cost-effective for production of industrial enzymes.

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