Punjab Univ. J. Zool., Vol. 31 (2), pp. 261-267, 2016
ISSN 1016-1597(Print)
ISSN 2313-8556 (online)

Original Article

Extracellular lipase production from *Bacillus subtilis* using agro-industrial waste and fruit peels

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(**Article history:** Received: November 16, 2016; Revised: December 23, 2016)

Abstract
This study was planned for the production of lipases by solid-state fermentation (SSF) using agro-industrial wastes as substrate**.** A total of 14 bacterial strains were isolated, screened and compared for lipase production. Among the evaluated strains, *Bacillus subtilis* strain NL-39 was selected on the basis of best lipase production. The maximum enzyme activity (34.93 U/ml) was shown by *B. subtilis* using SSF with soya bean meal. The lipase production by other low cost agro-industrial waste, including sunflower meal, wheat bran, oat bran, rice bran and sugar cane bagasse was 23.83, 12.17, 10.40 10.00 and 16.23 U/ml, respectively. The lipase production by *B. subtilis* using peels of different fruits, including banana, orange, water melon and melon as carbon source, was 27.17, 21.37, 10.57 and 8.43 U/ml, respectively. The corn cob produced 12.27 U/ml while waste oils of various industries produced 16.17 U/ml (Shan oil), 13.67 U/ml (automobile), 13.37 U/ml (unbranded waste cooking oil) and 6.03 U/ml (Sitara oil) of enzyme activity when used as substrate for lipase production. It is concluded that among the agro-industrial wastes, fruit peels and waste oil samples used in this study, the maximum lipase production was observed with soya bean meal, banana peel and Shan oil waste, respectively.
Key words: Enzyme production technology, lipase production, *B. subtilis*, solid state fermentation, different carbon sources, fruit peels, wheat bran, soya been waste, sunflower meal and oil industry waste.

To cite this article: MAZHAR, H., ABBAS, N., HUSSAIN, Z., SOHAIL, A. AND ALI, S.S., 2016. Extracellular lipase production from *Bacillus subtilis* using agro-industrial waste and fruit peels. *Punjab Univ. J. Zool.,* **31**(2): 261-267.

INTRODUCTION

Lipases (triacylglycerol acyl-hydrolases, EC 3.1.1.3) are the enzymes that catalyze the hydrolysis of ester bonds of triacylglyceroles, releasing free fatty acids, mono or diacylglycerol in the oil water interface (Treichel *et al*., 2010). Lipases may be of animal, microbial or plant origin, with variations in their catalytic properties and can be obtained by solid state fermentation (SSF) or by submerged fermentation (Annibale *et al*., 2006; Papagora *et al*., 2013). SSF includes the metabolism and growth of microorganisms without free water on a moist solid. This technique has been introduced in recent years, especially because of the advantage of using low cost agro-industrial residues with lower amounts of water, which releases negligible or considerably lower quantities of effluent, thus reducing pollution concerns (Azeredo *et al*., 2007). Current trends in SSF have concerned on the application of SSF for the production of valuable products such as biologically active secondary metabolites, including enzymes. Due to easy production along with inexpensive techniques of fermentation and low energy consumption, the lipases obtained from microbes (fungi, yeast and bacteria) are given preference for industrial application. However, SSF is most appropriate process due to its various benefits and bioconversion parameters. Lipase production by SSF using olive cake and sugarcane bagasse are very cost effective (Cordova *et al*., 1998) and in this case oil cakes show best production (Ramachandran *et al*., 2006). The agro-industrial residues have great potential for use as substrate in solid state

138-PUJZ-61026111/16/0261-0267
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fermentation, due to their low cost and high yield value products. This is due to their composition of organic matter which is easy to be consumed by microorganisms (Pelizer *et al*., 2007). For the production of lipases, several agro-industrial remains have revealed significant potential. These include sugarcane bagasse (Ellaiah *et al*., 2004; Pelizer *et al*., 2007), wheat bran (Martins *et al.,* 2002), soybean meal (Menoncin *et al.,* 2009) and castor bean cake (Godoy *et al*., 2009).The most useful lipase producer bacterial genus used in industry is *Bacillus.* They are gram positive endospore formers and produce large variety of enzymes and considered as strong extracellular lipase producer. They have demonstrated the ability to grow in various ranges of temperature, pH and salt concentration (Jaeger *et al*., 1994). Due to its stability the lipases have immense industrial importance and are used as additives in detergents used by households and industrial laundry. As biodetergent lipases must resist alkaline environment. Lipases of desired quality can be obtained by many trials (Yeoh *et al*., 1986). The lipase removes the oil stains from clothes and fabrics. They have the ability to change the location of fatty acid chains in the glyceride and replacing fatty acid with new one and enhance the flavor of cheese. Lipase catalyzed trans-esterification of fatty acid replacing the palmitic acid with stearic acid in palm oil. Lipases upgrade the undesirable fats in cocoa butter substitutes (Coleman and Macrae, 1980., Undurrage *et al.,* 2001).The lipase released from microbes has advantage to remove lipid contents and it is environment friendly treatment of waste water (Dharmsthiti and Kuhasuntisook, 1998). Cold adopted lipases have great potential in the field of waste water treatment in cold environments (Buchon *et al*., 2000). Lipases have been used in the synthesis of biopolymer. It is also used in recombinant DNA technology. Hence there is dire need to explore new bacterial strains and ways for production of lipases.The present work was designed for the cost effective production of lipases using locally isolated *Bacillus* species and low cost agro-industrial and other wastes through solid state fermentation.

MATERIALS AND METHODS

Collection of sample

Different sites were visited to collect the samples of soil and water which included oil industry, automobile garages and household wastes and numbered as 1, 2 and 3.

Isolation of lipase producing bacteria

Serial dilution technique (Akano and Atanda, 1990) was used to isolate the lipase producing bacterial strains from collected samples. Tributyrin-agar base medium was used for screening lipase producing bacteria. The appearance of transparent zone indicated the lipolytic activity resulting when the tributyrin was decomposed in the fatty acid. Tributyrin agar medium was composed $gl^{-1}$of: $(NH_4)_2SO_4$, 5; $CaCl_2$, 3; Na_2HPO_4, 6; $MgSO_4$, 3; KH_2PO_4, 2; agar 20 and tributyrin, 10ml with pH 6.0. Counting of appeared bacterial colonies was carried out with suspected lipase producing potential.

Media for lipase production

Medium (Liquid) for lipase production contained: gl^{-1} of glucose 5, peptone 5, yeast extract 5, $MgSO_4 7H_2O$ 0.5, NaCl 3 and olive oil 5%. After mixing the media was autoclaved for 20 min at 15 lbs pressure. Inoculums (2%) was added and incubated for 24 hours at 37 ^oC.

Phenol red agar plate assay

Phenol red agar plates were prepared using phenol red (0.01% w/v) along with olive oil, 2% v/v; agar, 1%: $CaCl_2$, 0.1% and pH was adjusted at 7.4. The solution was autoclaved at 121°C at 15 lbs pressure, cooled and poured in the sterile glass Petri plates aseptically (Singh *et al*., 2006). In order to obtain 5mm diameter the sterile agar plates were aseptically punched with a sterile cork borer. Freshly extracted crude enzyme (10 µl) was poured into phenol red agar plates and incubated for 24 hours at 37°C. For indicating the enzyme activity the change in phenol red color was used as benchmark. Phenol red agar well diffusion method was used in order to assay the crude lipase.

Physical and biochemical characterization of isolates

Physical and biochemical characterization of isolate: colony morphology, gram staining, sporulation test, motility test, Voges-Proskauer (V.P.) test, indole test, catalase test, oxidase test, coagulase test, urease test, tyrosine decomposition, starch hydrolysis, methyl red test, nitrate reduction, citrate utilization, growth at different temperature and growth at different pH (Singh *et al.,* 2006; Mazhar *et al.,* 2016).

16S rRNA gene sequencing

Overnight culture was inoculated into LB broth (g/l: tryptone 10, yeast extract 5 and NaCl5 and pH 7.0), in shaking incubator at 37°C for 24 hours. DNA was isolated using phenol/chloroform method (Sambrook *et al*, 1989). Following amplification procedure has been adopted for amplification.

Step 1- **Initial Denaturation**: at 94°C for 5 min
Step 2- **Denaturation:** 94°C for 1 min
Step 3- **Annealing**: 52°C for 1 min
Step 4- **Elongation:** 72°C for 1 min
Step 5- **Extension**: 72°C for 10 min final

Following DNA primers were used in this experiment:

Pro Forward: 5'-AAACTYAAAKGAATTGACGG-3
Pro Reverse: 5'-ACGGGCGGTGTGTRC-3'
Y = C/T; K = G/T; R = A/G

The forward primer was complementary to the upstream of 16S rDNA and the reverse was complementary to the upstream sequences of 23S rRNA gene sequences. A set of universal primer was used to amplify 16S rRNA gene. The sequence for 16S rRNA gene of the isolate was submitted in Gene Bank (Mazhar *et al.,* 2016).

Substrate selection

Different agro-industrial wastes were used for low cost lipase production like, 5% rice bran, wheat bran, sunflower meal, soya bean extract, oat bran, molasses, corn cob and sugar can bagasse. Oil wastes like Sitara oil industry waste, Shan ghee industry waste, automobile waste oil, unbranded waste cooking oil and fruit peels *e.g.*, orange peel, banana peel, melon peel, water melon peel were substrates for lipase production.Agro-industrial waste was dried under sun for three days and ground to achieve the desired size. The procurement of oil cake was made from a local market of Lahore. It was dried at room temperature to reduce moisture content (Ramachandran *et al*., 2006).

Inoculum preparation

Minimal medium includes gl^{-1}: NH_4Cl 2.0, Na_2HPO_4 6.0, $MgSO_4$ 0.1, NaCl 5.0, KH_2PO_4 3.0 was used to inoculate loop full of cells from freshly grown log phase culture (Oswal *et al*., 2002) and incubated in a shaking incubator at 30°C and 180 rpm for 24 hours.

Media for solid state fermentation (SSF)

For SSF 10g of different waste oils, agro-industrial wastes, and fruit peels in 250ml flask was over layered by 50ml of fermentation medium. The medium was composed of (gl^{-1}) diammonium hydrogen phosphate 1.0, magnesium sulphate $6H_2O$ 0.40, peptone 2.0, sodium chloride 2.50, calcium chloride 0.40, Tween-80 1–2 drops. The pH was set at 7.0 and temperature at 37˚C (Mobarak *et al*., 2011). Many sets of, 250ml Erlenmeyer flasks containing fermentation medium were inoculated with enriched cultures. The flasks were incubated at static conditions at 37˚C for 2 days.

Extraction of crude enzyme

Fermentation mixture was mixed with 10ml of 50mM phosphate buffer pH 7. This mixture was shaken for 30 min at room temperature in order to smoothen the process of extraction of enzymes. After extraction a double layered muslin cloth was used to squeeze through the suspension for 5minutes at 12,000 rpm (Falony *et al*. 2006).

Lipase activity assay

Standard titrimetric method was used for assaying lipase activity. Substrate for enzyme assay was prepared by emulsifying olive oil (10%v/v) with gumArabic (5%w/v) in 5ml of phosphate buffer pH 7 and 2ml of 0.6% $CaCl_2$ solution. During estimation 500µl of crude enzyme extract was added and substrate-enzyme mixture was incubated in rotary shaker at 150 rpm and 30˚C for 15 minutes. Ethanol solution: 3ml of acetone (1:1) was used for termination of reaction and withdrawal of fatty acids. For estimation of fatty acid, phenolphthalein was used as an indicator dye during titration of fatty acids with 0.1M NaOH at pH10.5. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1µmol of fatty acids/min from triglyceride substrate (tributyrin).

RESULTS

Samples collected from different sources were examined after serial dilutions. Lipase producing strains were isolated on tributyrin-agar plates on the basis of lipolytic activity. Fourteen bacterial isolates had potential for lipase production. The isolates showed clear zones of hydrolysis on tributyrin-agar plates, and selected as lipase producer.

Screening of lipase producing isolates

Different qualitative and quantitative methods of screening were applied for the selection of best lipase producers. The isolate strain NL-39 showed maximum lipase production (Table 1), which was used for further studies.

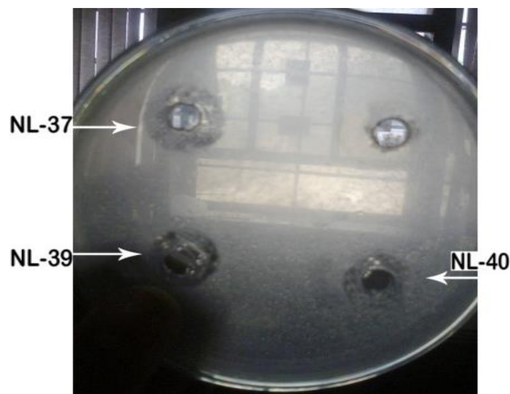

Figure 1: Zone of hydrolysis seen on tributyrin agar plates

Identification of selected isolate

Identification was carried out by morphological, biochemical characterization and rDNA sequencing (Table 2). It was identified as *Bacillus subtilis* on the basis of the sequence homology with the existing Gen Bank sequences. Sequence for the distinct rDNA fragment was submitted to Gene Bank and accession number assigned is KT374117.

Effect of various agro-industrial by-products on lipase production

Various agro-industrial byproducts like wheat bran, rice bran, sunflower meal, oat bran, soya bean extract and sugar cane bagasse were tested as a carbon source for their effect on lipase production (Figs.1, 2).

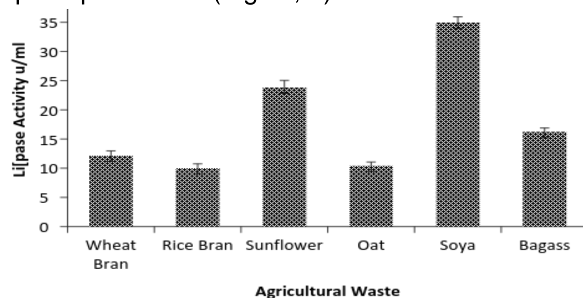

Figure 2: Lipase production by *Bacillus subtilis*-(N-39) using different agro-industrial waste

Inclusion of 2.5% soybean meal which is a byproduct of oil mills was found to be the best protein substrate for the induction of extracellular lipase. The maximum lipase activity after addition of soya bean extract was 34.93±1.08U/ml^{-1} showed by *B. subtilis* NL-39. Sunflower meal, however, produced 23.83 U/ml of lipase. Different waste oils were also used as substrate for lipase production by *B. subtilis* NL-39 which showed highest lipase activity (13.67U/ml) by using automobile waste. Early same lipase production was (13.37U/ml) observed with waste cooking oil whereas decline in lipase activity was noticed with Shan and Sitara industry wastes (Fig. 3). Peels of various fruits were used as substrate for lipase production for example orange, banana, melon, water-melon and corn cob. The isolate NL-39 showed maximum lipase production by utilizing banana (27.17 U/ml) and orange peels (Fig. 4).

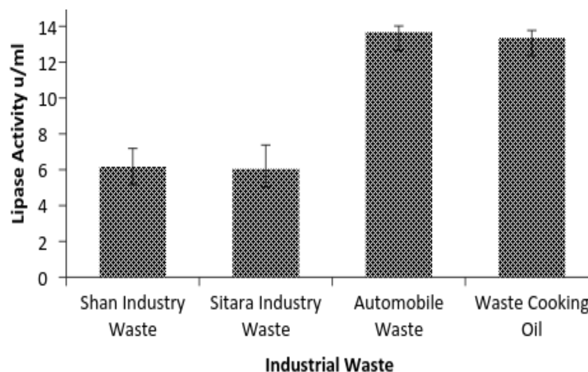

Figure 3: Lipase Production by *Bacillus subtilis*-(N-39) using different industrial oil waste

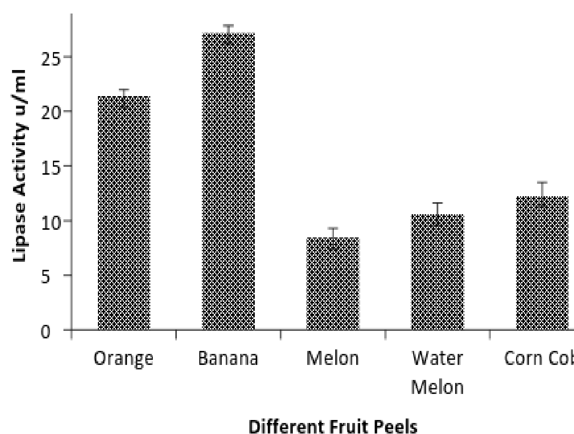

Figure 4: Lipase production by *Bacillus subtilis*-(N-39) using different fruit peels

Table I: Diameter of zones of hydrolysis shown by crude enzyme extracts of isolates on tributyrin-agar plates after 24 hours

Sr. No.	Isolates #	Diameter of zone of hydrolysis (mm; n=3)
1	NL 39	1.67 ± 0.12
2	NL 40	1.67 ± 0.05
3	NL 41	0.60 ± 0.10
4	NL 42	0.13 ± 0.05
5	NL 43	0.27 ± 0.05
6	NL 44	0.20± 0.03
7	NL 45	0.53 ± 0.06
8	NL 46	0.13 ± 0.05
9	NL 47	0.23 ± 0.04
10	NL 48	0.60 ± 0.01
11	NL 49	0.60 ± 0.17
12	NL 50	0.70 ± 0.00
13	NL 51	0.33 ± 0.02
14	NL 52	0.35 ± 0.01

The data has been mentioned as Mean ± SEM.

This isolate N-39 was a Gram +ve rod shaped, motile and spore former bacteria which showed growth in the range of 15ºC–60ºC and pH 5–8. NL-39 showed positive for oxidase, catalase, VP, nitrate reduction, casein, starch and gelatin hydrolysis tests while negative for indole and methyl red tests. The isolate also showed positive growth on **g**lucose and maltose while could not grow on sucrose**, f**ructose and manitol.

DISCUSSION

Solid state fermentation has gained attention in recent years due to the possibility of using low cost agro-industrial wastes as substrate. In this study, different agro-industrial wastes were used as substrate for lipase production. Present study reports the use of different agro-industrial wastes like wheat bran, rice bran, oat bran, soya bean meal, sugar cane bagasse and sunflower meal as a carbon source by bacteria for lipase production. Branded and unbranded oil waste was also used for this purpose. This study has also explored the possibility of using different fruit peels, like melon, water melon, oranges, banana and corn cob, as a carbon source for the lipase production by SSF using *Bacillus* spp. Different *Bacillus spp.,* and strains produce variable amounts of lipases under different temperature, pH and nutrional source (Mohan *et al., 2008).*

Among the various types of waste used in this study, relatively, higher levels of lipase activity was shown by soya bean meal, banana peel, sunflower meal and orange peel which indicates that these agriculture wastes contain all the necessary nutrients required for the growth of *Bacillus* spp., in balanced amounts. As the highest level of lipase activity was shown by soya bean meal, this substrate was used for further experimentation in this study with *B. subtilis.* De-Almeida *et al* (2016) has shown lipase production by various substrates individually and after mixing with oils like olive oil. According to this study the highest level of enzyme activity was observed with wheat bran; intermediate level was observed with barley spent grain and low or no enzyme activity was observed with citrus pulp, sugarcane bagasse and cassava peel when used with maximum 40% olive oil. In this study these carbon sources individually produce less enzyme activity. In another study comparing coconut oil and groundnut oil with different agro-industrial wastes, groundnut oil showed maximum extracellular lipase specific activity (Suji *et al*., 2014). Treichel *et al*. (2009) investigated the lipase production from soybean meal as substrate and the effect of different agro-industrial wastes (wheat bran, rice husk, lentil husk, banana, water melon and melon wastes) on lipase production by SSF using *Bacillus coagulans*. The best lipase production was obtained using solid waste from melon supplement with ammonium nitrate and 1% olive oil.The lipase produced by *B. subtilis* during this study had maximum activity at neutral pH. The findings in this study are in agreement with several other reports which showed the induction of lipases with best activity at neutral pH (Dharmsthiti*, et al.,* 1998; Dharmsthiti and Luchai, 1999; Lee, *et al.,* 1999) or alkaline optimum pH (Schmidt Donnert, *et al.,* 1994; Sidhu, *et al.,* 1998; Kanwar and Goswami, 2002). It has been showed that bacterial lipases are stable up to 60˚C. The lipase produced by *B. subtilis* in this study showed maximum activity at 40˚C which indicates decreased temperature tolerance of the *Bacillus* strain used in this study. Neethu *et al.,* (2015) has showed significantly higher amounts of lipases by a bacterium, *Stenotrophomonas maltophilia* using different industrial wastes singly like, groundnut cake (74.12. U/ml) followed by coconut cake (61.91 U/ml), neem cake (58.74. U/ml), wheat bran (49.61 U/ml) and rice bran (28.165.U/ml).The findings from different studies, as discussed above, have proved that not only the appropriate combination of different industrial wastes is required for higher lipase

production but pretreatment or processing of these materials (Kumar *et al.,* 2011) also play a significant role.

It is concluded from this study that *B. subtilis* strain (NL-39) isolated during this study showed a significantly good potential to utilize all the tested agro-industrial and industrial wastes for the low cost production of extracellular lipase. Soya bean meal, automobile waste, cooking oil waste, orange peel and banana peel showed more promising results, for the production of lipase. This study can be further extended and improved enzyme production can be achieved by using combinations of different nutrients.

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