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ORIGINAL PAPER

Effects of lipidic carbon sources on the extracellular lipolytic activity of a newly isolated strain of *Bacillus subtilis*

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Abstract An isolate exhibiting high extracellular lipolytic activity was identified as *Bacillus subtilis* by 16S rRNA gene sequence analysis. The enzyme activity of the isolate was improved by using different concentrations of lipidic carbon sources such as vegetable oils, fatty acids and triglycerides. Lipolytic activity was assayed spectrophotometrically using *p*-nitrophenyl palmitate. One percent (v/v) of sesame oil provided the highest activity with 80 and 98% enhancements with respect to 1% (v/v) concentrations of linoleic acid and triolein as the favored fatty acid and triglyceride, respectively. Glucose presented a repressive effect on lipase production. Lipase secreted by *B. subtilis* was partially purified by ultrafiltration and anion exchange chromatography; and the purified enzyme was tested for its residual activity in the presence of EDTA, SDS, Triton X-100, Tween 20, Tween 80 and protease. The present work reports, for the first time, that the lipolytic activity of a *B. subtilis* strain can be improved by using inexpensive vegetable oils; and also that *B. subtilis* lipase is suitable for use in detergents.

Keywords *Bacillus subtilis* · Lipolytic activity · Lipase · Vegetable oils

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Introduction

Lipolytic enzymes, i.e. lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1), produced by microorganisms are of great interests due to their biotechnological potential. They are widely used in detergents, textile and dairy industries; oil processing; production of surfactants; and synthesis of chiral pharmaceuticals. Following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume [10]. Lipolytic enzymes are produced by widespread of microorganisms such as bacteria, fungi, yeasts and actinomyces; and their features differ as regard their origins. Therefore, attempts to isolate microorganisms that facilitate the discovery of novel lipases always gain attention. Among bacterial lipases being exploited, those from *Bacillus* display properties that make them promising candidates for biotechnological applications [16]. *B. subtilis, B. pumilus, B. licheniformis and B. alcalophilus* are among common bacterial lipase producers [9]. Lipolytic enzymes produced and secreted by *B. subtilis* are of substantial biotechnological interest and many have, therefore, been identified, cloned, and characterized [6–8].

Microbial lipase production involves the use of a lipid or related substance (triglyceride, fatty acid and detergent, etc.) alone or jointly with sugars as a carbon source and/or as an inducer of the production [18]. From the industrial point of view, utilization of natural nutrients to form cost-effective production media is attractive. *Bacillus* can efficiently utilize complex nutrient sources [8]. However, the literature reports on the development of a medium composed of natural nutrients such as vegetable oils for high lipolytic activity of isolated *Bacillus* species are limited [13, 15, 17]; and, to our knowledge, none of them reports on *B. subtilis*.

This work describes an investigation on the enhancement of extracellular lipolytic activity of an isolate, which was identified as *B. subtilis* by 16S rRNA gene sequence analysis, by using different concentrations of vegetable oils, fatty acids and triglycerides. The utilization of sesame oil provided the highest lipolytic activity indicating that inexpensive carbon sources are good candidates to produce lipase enzyme from *B. subtilis*. Glucose was also used as an alternative substrate; however, it presented a repressive effect on lipase production. Partial purification of the lipase and testing its compatibility with detergent additives were also performed.

Materials and methods

Chemicals

The chemicals were of reagent grade and purchased from commercial suppliers (Merck, Sigma-Aldrich). The vegetable oils were supplied from the market and analyzed for their fatty acid compositions [3] after converting the fatty acids to their methyl esters [19]. The results are given in Table 1.

Strain isolation

Lipase producing microbial cultures were isolated from soil and enriched by periodic sub-culturing of samples in nutrient broth (NB) containing 1.0% (v/v) tributyrin. The composition of NB medium is 0.5% (w/v) peptone and 0.3% (w/v) beef extract. The isolation process was performed by serial dilution of samples on tributyrin agar (TBA) plates according to standard techniques [12]. The composition of the TBA medium is 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.1% (v/v) tributyrin and 1.5% (w/v) agar. *Bacillus* sp. was selected on the basis of producing the largest opaque hole. Active colonies were re-streaked on TBA agar for purification.

Identification of the isolate

Cell morphology, Gram staining and spore production properties of the isolated lipase producing bacterial culture were determined by standard techniques [4].

Amplification of DNA of the isolate was carried out with the BioRad DNA Engine Tetrad 2 thermal cycler (USA) under the following conditions: 94 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; final extension at 72 °C for 10 min. 1,525 bp 16S rRNA sequence was amplified as one single fragment using universal primer pair (F-CCG TCG ACA GAG TTT GAT CCT GGC T; R-GGC TAC CTT GTT ACG ACT T). Sequencing reactions were carried out using this amplification primers as well as additional universal, internal primers (F1-AGG GTC ATT GGA AAC TGG G; F2-AGG ATT AGA TAC CCT GGT AGT CCA; R1-CCC AGT TTC CAA TGA CC; R2-CGT GTT GTA GCC CAG GTT A). Sequencing reactions were setup using Beckman DTCS quick start kit. Purified sequencing reactions were run on Beckman CEQ 8000 Genetic Analysis System (USA).

Culture media and conditions

The slant cultures were maintained on TBA medium. The microorganism incubated at 30 °C for 24 h was inoculated into 10 ml pre-culture medium of the following composition: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1.0% (v/v) tributyrin in phosphate buffer (0.1 M pH 7.5). After incubation at 37 °C and 150 rpm conditions for 24 h in an orbital shaker (Edmund Bühler SM-30, Germany), the cells were transferred into 100 ml enzyme production medium. Lipase production was carried out in shake flasks where different lipidic substances were used to find out the substrate for the highest lipolytic activity. The standard operating conditions, which were determined previously, were the following: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, initial pH 7.5 provided by 0.1 M phosphate buffer, temperature 37 °C and stirring rate 150 rpm.

Enzyme assays

Lipolytic enzyme activity was measured spectrophotometrically (Shimadzu 1601A) using 0.5% (w/v) *p*-nitrophenylpalmitate in ethanol as substrate. The procedure given by Hung et al. [11] was modified as follows: 1 ml substrate was added to 1 ml phosphate buffer (0.05 M pH = 8) containing 0.1 ml sample and stirred for 5 min at 30 °C. The

Table 1 Fatty acid composition of commercial vegetable oils

	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0
Sesame oil	5.959	0.108	3.632	35.995	53.032	0.115	0.297	0.189	0.673
Sunflower oil	6.024	0.086	3.388	26.157	63.335	0.075	0.143	0.128	0.663
Soybean oil	13.798	0.136	3.396	25.059	51.208	5.623	0.313	0.248	0.219
Corn oil	12.199	0.172	1.822	27.679	56.777	0.859	0.296	0.194	–
Olive oil	12.312	0.815	2.698	73.383	9.207	0.638	0.454	0.325	0.168

hydrolytic reaction was terminated by adding 2 ml 0.5 N Na_2CO_3 followed by centrifugation at 12,000×*g* (Hettich Rotina 35R, DJB Labcare, UK) for 10 min. The absorbance at 404 nm was determined. One unit enzyme activity (U) was defined as the amount of enzyme necessary to produce 1 μmol *p*-nitrophenol from *p*-nitrophenylpalmitate per min under the conditions mentioned above.

Alkalimetric final titration for lipolytic activity was also used in some experiments. The method by Cernia et al. [2] was adapted as follows: The mixture, containing 2.5 ml phosphate buffer solution (0.1 M pH 7.2), 0.5 ml olive oil and 0.1 ml sample was incubated at 37 °C under magnetic stirring for 30 min. After terminating the reaction with 2.5 ml acetone:ethanol mixture 1:1 (v/v), the solution was titrated with 0.1 M NaOH in the presence of phenolphthalein as indicator. One unit lipase activity (U) was defined as the amount of enzyme that catalyzes the release of fatty acid per min under the conditions mentioned above.

For intracellular lipolytic activity assay, cells were harvested by centrifugation at 12,000×*g* for 10 min at 4 °C, washed in phosphate buffer solution (0.1 M pH = 7.5) and resuspended to a 2 ml final volume with the same buffer. The cell suspension was disrupted with a sonicator (Sonics Vibra Cell VC 130) with a power input to 60 W and a frequency of 20 kHz for two periods of 20 s. The disrupted cells were centrifuged at 12,000×*g* at 4 °C for 10 min and the supernatant was used as the cell extract for the determination of intracellular activity.

Protease activity was assayed spectrophotometrically (Shimadzu 1601A, Japan) using casein as substrate [20]. One unit of enzyme activity is defined as 4 nmol tyrosine released per min per ml.

All experiments were carried out in duplicate and the activities given throughout the paper represent the average of at least three determinations. The standard deviations were within ±5%.

Protein analysis

Protein concentration was measured spectrophotometrically (Shimadzu 1601A, Japan) at 595 nm by the Bradford method using bovine serum albumin as protein standard [1].

Enzyme purification

The enzyme was partially purified by ultrafiltration and anion exchange chromatography. Cells were removed from the culture medium by centrifugation at 12,000×*g* for 20 min at 4 °C. The cell-free supernatant was concentrated on Amicon Ultra-15 (5 kDa cutoff) centrifugal filter units (Millipore, USA). The precipitated sample was dissolved in Tris–HCl buffer (20 mM pH = 8.3) and then applied to Bio-Rad UNO Q1 column (Bio-Rad Duo Flow System, USA) for anion exchange chromatography pre-equilibrated with a loading buffer of Tris–HCl (20 mM pH = 8.3) with a flow rate of 1.0 ml/min. The column was eluted with a linear gradient of the same buffer containing 1 M NaCl with a flow rate of 1.0 m/min. The fractions (0.4 ml) were collected using a fraction collector (Bio-Rad BioFrac Fraction Collector, USA); and those showing lipolytic activity were pooled. The protein content and lipolytic activity of the fractions were measured.

Results

Identification of the isolate

The isolated strain was an aerobic, endospore forming, Gram positive, motile, rod-shape bacterium belonging to the genus *Bacillus* sp. The nucleotide sequence (5X coverage) was analyzed against all 16S rRNA sequences on GenBank using blastn. The 16S rRNA sequencing of the isolate showed highest homology (99%) with *B. subtilis* thus it is classified as a variant of *B. subtilis* [5].

Effect of fatty acids

The effects of fatty acids on the lipolytic activity of the isolated *B. subtilis* were investigated by adding 1.0% (v/v or w/v %) concentrations of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) in the enzyme production medium. The highest extracellular activity was obtained with linoleic acid (0.032 U/ml), which was followed by oleic acid, palmitic acid and stearic acid, subsequently (Fig. 1). The time required to reach the highest lipolytic activity was lower for linoleic acid (24 h) than for the other fatty acids. The results indicated that as the number of double bonds in fatty acids increased, *B. subtilis* exhibited higher lipolytic activity. However, no significant difference was observed between the activities obtained in the presence of stearic acid and palmitic acid, which are saturated fatty acids of different chain lengths.

Low concentrations of a carboxylic acid, i.e. acetic acid, were also used as substrate. 0.1% (v/v) acetic acid induced higher lipolytic activity (0.054 U/ml at 54 h) than the fatty acids induced (Fig. 1). However, as the medium became acidic (pH = 4.6) by introducing 0.5% (v/v) acetic acid, higher concentrations of acetic acid were not examined. In the present study, all experiments were carried out at the initial pH of 7.5 which was previously determined to be the optimal value (data not shown). Therefore, the reason for the low activity obtained at 0.5% (v/v) acetic acid concentration was the low initial pH value.

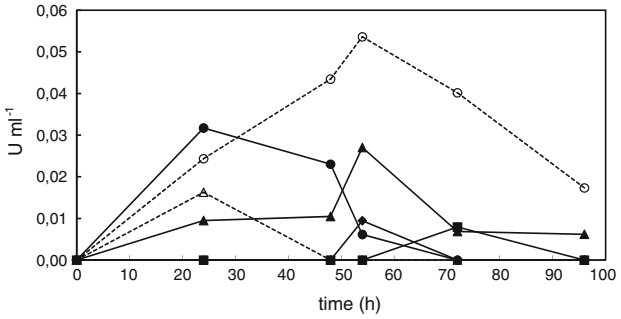

Fig. 1 Effect of fatty acids on the extracellular lipolytic activity of isolated *B. subtilis.* Acetic acid (C2:0) 0.1% (*open circle*), acetic acid (C2:0) 0.5% (*open triangle*), palmitic acid (C16:0) 1.0% (*filled diamond*), stearic acid (C18:0) 1.0% (*filled square*), oleic acid (C18:1) 1.0% (*filled triangle*), linoliec acid (C18:2) 1.0% (*filled circle*) [conditions: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, pH = 7.5, T = 37 °C, N = 150 rpm, enzyme activity was assayed with PNPP]

Effect of triglycerides

The effects of triacetin (C2:0), tributyrin (C4:0), tricaprylin (C8:0), tripalmitin (C16:0), tristearin (C18:0) and triolein (C18:1) on the lipase production by the isolated *B. subtilis* was investigated by adding 1.0% (v/v) concentrations of triglycerides in the enzyme production medium. The highest extracellular activity was obtained in the presence of triolein at 54 h as 0.046 U/ml (Fig. 2). Lipolytic activity decreased as the chain length of triglyceride became shorter except for low chain length triglycerides, namely triacetin and tributyrin. The unsaturated triglyceride (triolein) induced higher activity than the saturated triglyceride of the same chain length (tristearin). On the other hand, for all types of triglycerides, a decrease in the lipolytic activity was observed at high cultivation times. This was due to protease activity, which was determined in the culture medium at 96 h as 8.30, 7.29, 4.68 and 7.59 U/ml in the presence of triolein, tricaprylin, tristearin and tripalmitin, respectively.

The comparison of extracellular lipase activities obtained in the presence of fatty acids and triglycerides showed that triglycerides yielded higher lipolytic activities than the corresponding fatty acids except for triacetin (Fig. 3). This result can be addressed to the additional induction effect of glycerol that is produced by the hydrolysis of triglycerides in the enzyme production medium.

Effect of vegetable oils

The effects of olive, corn, sunflower, soybean and sesame oils as natural nutrients on the lipolytic activity of the isolated *B. subtilis* were investigated for their 1.0% (v/v) concentrations in the enzyme production medium. *B. subtilis* produced lipase in the presence of all vegetable oils considered in this study. The results showed that the extracellular activity decreased in the presence of vegetable oils following the order: sesame oil (0.155 U/ml) > sunflower oil (0.045 U/ml) > olive oil (0.036 U/ml) > corn oil (0.033 U/ml) > soybean oil (0.021 U/ml) (Fig. 4). The time to achieve the highest enzyme activity was dependent on the vegetable oil used. However, for all type of vegetable oils, a decrease in the lipolytic activity was observed at high cultivation times probably due to protease activity.

Lipolytic activity seemed to be closely related to the linoleic acid (C18:2) and oleic acid (C18:1) percentages in vegetable oils (Table 1). Linoleic acid could be considered to play the most important role in the induction of lipase, since it was the best fatty acid among others for lipolytic activity (Fig. 1). On the other hand, low percentages of medium chain length fatty acids in sesame oil and sunflower oil (Table 1) could be another reason for obtaining higher lipolytic activities with these substrates than other vegetable oils. Higher enzyme activities obtained with longer chain length fatty acids (Fig. 1) also implied that the

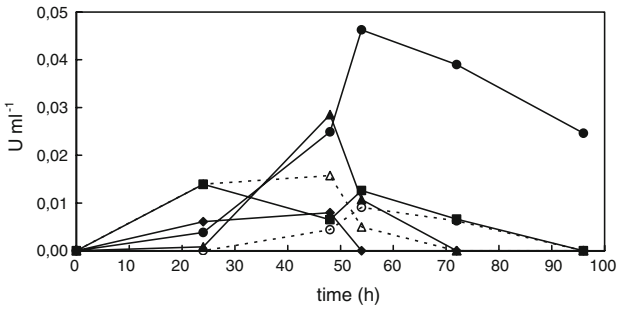

Fig. 2 Effect of triglycerides on the extracellular lipolytic activity of isolated *B. subtilis.* Triacetin (C2:0) 1.0% (*open triangle*), tributyrin (C4:0) 1.0% (*open circle*), tricapryln (C8:0) 1.0% (*filled diamond*), tripalmitin (C16:0) 1.0% (*filled square*), tristearin (C18:0) 1.0% (*filled triangle*), triolein (C18:1) 1.0% (*filled circle*) [conditions: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, pH = 7.5, T = 37 °C, N = 150 rpm, enzyme activity was assayed with PNPP]

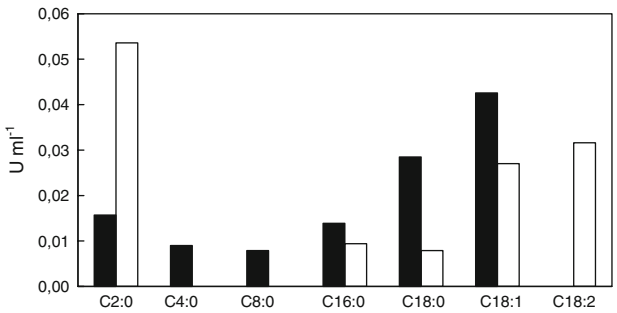

Fig. 3 Comparison of maximum extracellular lipolytic activities obtained in the presence of fatty acids and triglycerides. Triglycerides 1.0% (*filled square*), fatty acids 1.0% (*open square*) [conditions: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, pH = 7.5, T = 37 °C, N = 150 rpm, enzyme activity was assayed with PNPP]

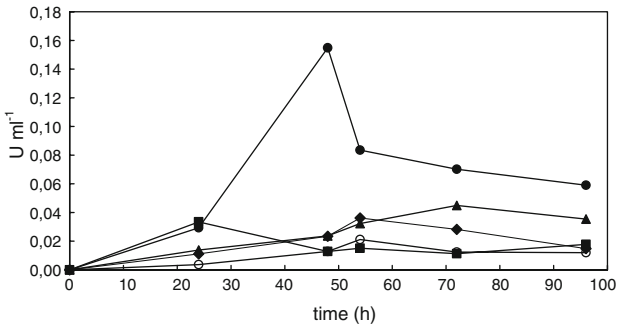

Fig. 4 Effect of vegetable oils on the extracellular lipolytic activity of isolated *B. subtilis*. Sesame oil 1.0% (*filled circle*), sunflower oil 1.0% (*filled triangle*), olive oil 1.0% (*filled diamond*), corn oil 1.0% (*filled square*), soybean oil 1.0% (*open circle*) [conditions: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, pH = 7.5, T = 37 °C, N = 150 rpm, enzyme activity was assayed with PNPP]

high percentages of long chain length fatty acids favored the lipolytic activity.

As sesame oil induced more extracellular lipolytic activity compared to other carbon sources, the effect of different concentrations of sesame oil (0.25, 0.5, 0.75, 1.0 and 1.5% v/v) was further studied to determine the amount of sesame oil required for maximum enzyme production from *B. subtilis*. The increase in sesame oil concentration from 0.25 to 1.0% (v/v) increased the lipolytic activity and decreased the time required to reach the highest activity (Fig. 5). The maximum activity was obtained at 1.0% (v/v) sesame oil concentration (0.1548 U/ml at 48 h). However, a further increase to 1.5% (v/v) concentration decreased the activity probably due to either insufficient contact of aqueous and oil phases related to ineffective mixing or substrate inhibition. Similar decrease at 1.5% (v/v) concentrations of other vegetable oils, fatty acids and triglycerides was also observed (data not shown).

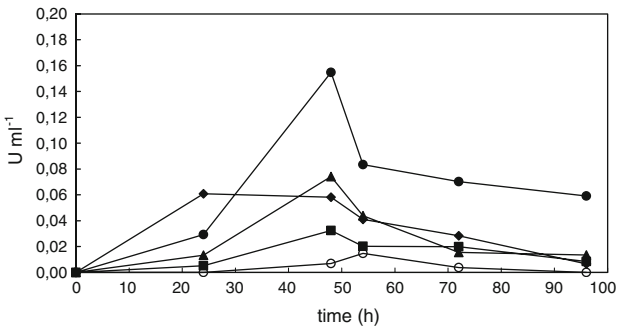

Fig. 5 Effect of sesame oil concentration on the extracellular lipolytic activity of isolated *B. subtilis*. 0.25% (*open circle*), 0.5% (*filled square*), 0.75% (*filled triangle*), 1.0% (*filled circle*), 1.5% (*filled diamond*) [conditions: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, pH = 7.5, T = 37 °C, N = 150 rpm, enzyme activity was assayed with PNPP]

Effect of glucose

The effect of glucose on the lipolytic activity of the isolated *B. subtilis* was investigated by adding 0.1, 0.5 and 1.0% (w/v) concentrations of glucose in the enzyme production medium containing 1.0% (v/v) sesame oil. The variations in extracellular lipolytic activities obtained in the presence of glucose in comparison with those achieved in the presence of sesame oil as a sole carbon source are shown in Fig. 6. Glucose presented in the enzyme production medium repressed lipolytic activity for all concentrations considered and the degree of repression was higher at high concentration of glucose.

Partial purification of lipase

Lipase secreted by *B. subtilis* was purified for preparing relatively pure form of the enzyme for testing its compatibility with detergents. The purification results are summarized in Table 2. The lipase was partially purified 13.3-fold with the final specific activity of 1,333 U/mg. Comparison of the specific activities towards PNPP and olive oil before and after purification showed that both assays gave similar results in terms of the fold purification.

Effect of detergents on purified lipase activity

The partially purified lipase was incubated with protease, Tween 20, Tween 80, Triton X-100, EDTA and SDS at the concentrations of 0.1% (w/v or v/v) for 1 h at 45°°C and 100 rpm, followed by the measurement of residual activity.

Table 3 displays the effects of detergents on the lipolytic activity. SDS resulted in complete inhibition of activity. EDTA also strongly inhibited the enzyme. Protease and Triton X-100 did not inhibit the enzyme significantly. Lipase lost only its half activity in the presence of Tween

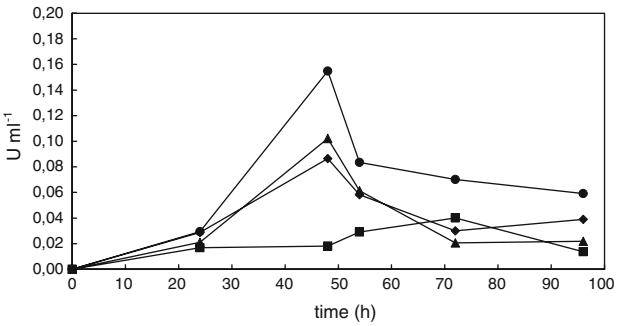

Fig. 6 Effect of glucose concentration on the extracellular lipolytic activity of isolated *B. subtilis*. Sesame oil 1.0% (*filled circle*), sesame oil 1.0% + glucose 0.1% (*filled square*), sesame oil 1.0% + glucose 0.5% (*filled triangle*), sesame oil 1.0% + glucose 1.0% (*filled diamond*) [conditions: 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, pH = 7.5, T = 37 °C, N = 150 rpm, enzyme activity was assayed with PNPP]

Table 2 Partial purification of the extracellular lipase produced by isolated *Bacillus subtilis*

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture sudstpernatant	190	2,532.7	25.27	100.2	100	1
Ultrafiltration	5	100	1.155	86.6	3.94	0.86
Anion exchange chromatography	1.2	15.99	0.012	1,333	0.63	13.3

Enzyme activity was assayed with olive oil

Table 3 Effect of detergents on the residual activity of the partially purified lipase produced by isolated *Bacillus subtilis*

Detergent (1.0%)	Residual activity (%)
Protease	75
Tween 20	50
Tween 80	50
Triton X-100	75
EDTA	0
SDS	25

Enzyme activity was assayed with olive oil

20 and Tween 80. These results indicated a potential utilization of the lipase secreted by the isolated *B. subtilis* in detergents.

Discussion

In the present work, we improved the medium composition that stimulates higher extracellular lipolytic activity of the isolated *B. subtilis* by using lipidic carbon sources. We have found that vegetable oils could be preferably used as inducers and carbon sources instead of fatty acids or triglycerides. Sesame oil provided the highest activity with 80 and 98% enhancements with respect to linoleic acid and triolein as the favored fatty acid and triglyceride, respectively. The highest lipolytic activity exhibited by isolated *B. subtilis* was 0.155 U/ml (0.416 U/mg protein).

Bacillus subtilis produces several extracellular hydrolytic enzymes and among them a phospholipase, a lipase LipA and an esterase LipB have been identified [8]. Eggert et al. [7] claimed that LipA and LipB of wild-type *B. subtilis* 168 were differentially expressed depending on the composition of the growth medium: LipA was produced in rich (LB supplemented with glucose) and in minimal medium (SMS supplemented with tryptophan and glucose), whereas LipB was present only in rich medium. The authors reported that the extracellular activity reached 12–15 U/L in rich medium and to 15–18 U/L in minimal medium. The lipolytic activity achieved with sesame oil in the present work (155 U/L) was higher than these values. On the other hand, Meghji et al. [14] reported that *B. subtilis* NRRL 365 produced high extracellular carboxyl esterase activity in submerged culture media containing wheat bran, corn steep liquor, and salts. Supplementation of this medium with glucose reduced esterase activity to 37% of that in the unsupplemented control. In the present work, we have found that glucose repressed the lipolytic activity of the isolated *B. subtilis* to a greater degree (up to 88%) depending on its concentration in the cultivation medium containing sesame oil.

Intracellular lipolytic activities of the isolated *B. subtilis* induced by tributyrin, triolein, sesame oil, corn oil, soybean oil, and glucose + sesame oil were also assayed with PNPP after 96 h of cultivation. The highest intracellular activity was obtained in the presence of 0.5% (v/v) corn oil (0.011 U/mg dw) followed by 1.0% (v/v) triolein (0.008 U/mg dw); however, the level of activities were significantly low. Although it is known that *B. subtilis* possessed intracellular carboxylesterase [14], the low or negligible activity associated with homogenized cells indicated that lipolytic activity of the isolated *B. subtilis* was predominantly extracellular.

Bacillus subtilis is an important bacterium that is used in industrial enzyme production. However, it is widely used in protease production rather than lipolytic enzymes. In the present study, we carried out a parametric study to enhance the extracellular lipolytic activity of a newly isolated *B. subtilis* by developing a production medium composed of cost-effective carbon sources. We showed, for the first time, that *B. subtilis* utilized natural lipidic sources more preferably than simple carbon sources to exhibit high lipolytic activity; and vegetable oils can be used successfully as economically viable substrates for lipase production. The newly isolated *B. subtilis* was also shown to have a potential of being utilized in enzyme industry such as in detergents when cultured in the medium proposed in the present study.

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