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Optimization and Partial Purification of an Extracellular Esterase Produced by a *Bacillus safensis* Strain Isolated from Ear Wax

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Abstract: The esterases catalyzing the hydrolysis of acyl esters are highly desired in industrial applications. An extracellular esterase producing, mesophilic and alkaliphilic Gram positive bacterial isolate obtained from human ear wax (sebum) was identified as *Bacillus safensis* strain. The *B. safensis* strain produced relatively higher amount of esterase after 36 h at 37°C under shaking conditions when coconut oil (1.75 %, v/v) and gelatin (0.6 %, w/v) were used in the Mineral-Based broth as carbon and nitrogen source, respectively. The esterase production by *B. safensis* strain increased by ~208 % by consecutive optimization of physico-chemical parameters. The esterase was partially purified by acetone precipitation. Enzyme showed greater affinity towards a relatively short C-chain ester p-NPA as substrate. The K_m and V_{max} values of the esterase were found to be 0.127 mM and 8.82 U/ml/min, respectively. The esterase was purified up to 2.8 fold by acetone precipitation (20-40 % saturation). The esterase was found to be a heptameric protein of 49 kDa (under native-PAGE) comprising seven units of 4 7 kDa as revealed by SDS-PAGE.

Key words: *Bacillus safensis*; extracellular; esterase; ear wax, purification; characterization; SDS-PAGE.

Introduction

Lipolytic enzymes including esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are carboxylic ester hydrolases that catalyze the cleavage and formation of ester bonds [3,6]. A wide range of different esterases exist that differ in their substrate specificity, their protein structure, and their biological function. Esterases (EC 3.1.1.1 and EC 3.1.1.2) preferably catalyze the hydrolysis of carboxyl ester linkages composed of short-chain fatty acids only in aqueous solution, and they can also catalyze the ester synthesis and trans-esterification in water free or water-restricted medium [11]. The hydrolase family of enzymes consists of two major types of enzymes namely lipases and esterases. Lipases and esterases can be distinguished on the basis of their substrate spectra, as esterases catalyze the hydrolysis of carboxylic ester bonds of short chain fatty acids (< 10 carbon atoms) while true lipases have marked preference for long chain fatty acids (>10 carbon atoms) as substrates [13]. Esterases produced by bacterial strains present in human sebum can be a very interesting subject of the studies.

Sebum secreted by the sebaceous gland in humans, is primarily composed of triglycerides (~41 %), wax esters (~26 %), squalene (~12 %) and free fatty (~16 %) acids [27]. The composition of sebum varies across species [5]. Wax esters like squalene, are unique to sebum and are not produced anywhere else in the body [24]. The bacterial species capable of utilizing sebum by producing esterases or lipases should be present in the sebum. Lipase/ esterase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairy waste, industrial wastes,

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oil seeds, decaying food, compost heaps, coal tips and hot springs [15,14]. A strain of *Bacillus* sp. was isolated from soil that produced an extracellular acetylesterase involved in decolorization of triphenylmethane dyes [22]. The demand for the biocatalysts with novel and specific properties such as specificity, stability, pH and temperature is increasing day by day [4]. Esterases have attracted interest because of their broad industrial value(s) such as chemical manufacture, agriculture and foods [11]. Esterases have been used to modify the physicochemical properties of triglycerides for organic synthesis reactions [9] and as a chiral biocatalyst they are useful for production of pharmaceuticals through the reactions involving various amines and primary or secondary alcohols. Stereo specific nature of esterase is also used for resolution of the racemic mixtures in numerous aspects of organic chemistry [20]. By producing esterase(s) with special properties, they offer numerous biotechnological, industrial and environmental applications in fine chemistry, detergent industry, cosmetics, pharmaceutical industries, biodiesel production and in sewage treatment [12,10]. In view of the above facts, there is a great need to explore novel lipases/ esterases from diverse microbial sources for potential industrial applications. The present study was undertaken to isolate an extracellular esterase producing bacterial strain from sebum *i.e.* ear was to explore the effect of physico-chemical parameters on production of esterase by *B. safensis* strain, its partial purification and characterization.

Materials and methods

Chemicals and reagents

$NaNO_3$, K_2HPO_4, $MgSO_4$, KCl, $FeSO_4.7H_2O$, tributyrin (S.D, Fine Chem Ltd., Hyderabad, India); yeast extract, gelatin, *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl laurate (*p*-NPL), *p*-nitrophenyl myristate (*p*-NPM), *p*-nitrophenol, beef extract, casein, peptone, tryptone, sodium dodecyl sulfate (SDS), *N*,*N*,*N*',*N*'-tetramethyl ethylenediamine (TEMED), ammonium persulfate, 2-mercaptoethanol, hydrochloric acid, and bromophenol blue (Merck Ltd., Mumbai, India); acrylamide, bisacrylamide (N,N'-methylenebisacrylamide), glycerol, glycine and Tris (2-hydroxymethyl-2-methyl-1, 3-propanediol) (Sigma Chemicals Co., USA) were used in the present study. All the chemicals used in present investigation were of analytical grade and high purity.

Isolation and screening of esterase producing bacteria

The esterase producing bacterial strain was isolated from sebum samples of different individuals using enrichment method. The isolation of esterase producing bacterial strains was done on the basis of qualitative and quantitative screening. Qualitative screening was done on Mineral based (MB) agar (pH 7.5) plates containing $NaNO_3$ (0.3 %, w/v), K_2HPO_4 (0.01 %, w/v), $MgSO_4.7H_2O$ (0.06 %, w/v), KCl (0.06 %, w/v), $FeSO_4.7H_2O$ (0.001 %, w/v), yeast extract (0.5 %, w/v) and Tributyrin (1 %, v/v) that were inoculated with each of the bacterial strains and observed for the formation of zone of hydrolysis around bacterial colonies after 36 h incubation at 37^0C. Quantitative measurement of esterase activity was done by a colorimetric assay method using crude esterase [9]. The hydrolyzing efficiency of the bacterial strain for substrate *p*-NPA was calculated as follows;

$$\text{Hydrolyzing efficiency (\%)} = \frac{Z - C}{C} \times 100$$

Where, Z: Diameter of solubilization zone; C: colony diameter

Production of crude extracellular esterase

The seed culture was grown in the nutrient broth at 37^0C with continuous shaking at 160 rpm for 36 h. Enzyme production was carried out by inoculating MB broth, containing gelatin (0.6 %, w/v) instead of yeast extract and coconut oil (1.75 %, v/v instead of tributyrin), with 2 % (v/v) of seed culture at 37^0C with continuous shaking at 160 rpm for 36 h. The culture broth was centrifuged at 10,000 x g for 10 min at 4^0C and the cell free broth was used as crude enzyme for esterase activity.

Assay of esterase enzyme

Esterase activity was measured as the ability of

the enzyme to hydrolyze *p*-nitrophenyl acetate (*p*-NPA) as reported previously [8]. The assay system contained 0.05 M Tris buffer (pH 8.0), 10 mM *p*-NPA and cell-free broth of bacterial isolate. Buffer and substrate were pre-incubated for 10 min at 40°C before adding crude esterase. The reaction mixture was incubated for 15 min (under shaking) at 40°C and absorbance readings were taken at 410 nm using UV-visible spectrophotometer after stopping the reaction by chilling at -40°C. Standards were run with appropriate concentrations of *p*-nitrophenol (*p*-NP). One unit of esterase activity is defined as amount of enzyme required to release one micromole of *p*-nitrophenol from *p*-NPA under standard assay conditions. Protein estimation was done by Lowry protein assay using Bovine serum albumin as a reference protein [18]. The bacterial strain showing the maximum extracellular esterase activity as indicated by the Hydrolyzing efficiency (%) was selected for further studies.

Identification of esterase producing bacterial isolate 'S8'

Molecular identification based on 16S rRNA gene sequencing, of bacterial isolate 'S8' was performed by Xcelris Labs Limited, Ahmadabad, India. The identity of the isolate was determined through a BLAST search and by constructing a phylogenetic tree using Neighbour-joining method. The phylogenetic tree presented a close similarity of bacterial strain 'S8' to *Bacillus* sp. Further morphological and biochemical characterization of esterase producing bacterial isolate 'S8' was done in accordance with the Bergey's Manual of Systematic Bacteriology that identified bacterial isolate 'S8' as a *Bacillus safensis* strain.

Esterase production by optimizing physico-chemical parameters

Various physico-chemical parameters were studied in order to achieve maximum enzyme production *B. safensis*. The optimal incubation time for the production of extracellular esterase by *B. safensis* strain was determined by incubating the inoculated MB broth set to pH 7.5 (2 %, v/v inoculum) at 37°C for the time intervals of 12 h, 24 h, 36 h, 48 h and 60 h under shaking (160 rpm). The cell-free culture broth after centrifugation at 10,000x g for 10 min (4°C) was assayed for esterase activity. The effect of various oils as carbon source was studied by growing *B. safensis* strain in MB broth containing different oils (1.0 %, v/v) such as castor oil, coconut oil, cotton seed oil, mustard oil or olive oil. The effect of concentration of oil/ carbon source on esterase production was studied with the addition of various concentrations (0.75 - 2.0 %, v/v) of coconut oil to MB broth. The broader composition of other nutrients in the MB broth was maintained constant. The effect of various organic nitrogen sources (0.5 %, w/v) like beef extract, casein, gelatin, peptone, tryptone or yeast extract was studied. Different concentrations of best nitrogen source (gelatin) such as 0.2 %, 0.3 %, 0.4 %, 0.5 %, 0.6 %, 0.7 % and 0.9 % (w/v) were used in the MB broth set to pH 7.5 and the inoculated broths were incubated at 37°C for 36 h under shaking (160 rpm). Effect of incubation temperature on esterase production was analyzed with a range of 25 to 45°C with 5°C interval. The effect of inoculum size was evaluated using different volumes of bacterial seed culture such as 1 %, 2 %, 3 %, 4 % and 5 % (v/v) to inoculate the production broth. Effect of different medium components was observed by inoculating different broths such as Luria Bertani broth, Muller-Hinton broth and Nutrient broth set to pH 7.5 using *B. safensis* strain and incubating at 37°C for 36 h under shaking (160 rpm). In each case, the cell-free culture broth was assayed for esterase activity.

Partial purification and biochemical characterization of esterase of *B. safensis* strain

Cell-free broth (crude enzyme) obtained after centrifugation at 10,000X g (4°C) for 15 min was precipitated using acetone. To 120 ml of crude enzyme at 4°C, chilled acetone was added slowly with continuous gentle stirring to achieve 0-60 % saturation and the contents were kept for 2 h at 4°C (with continuous stirring) to achieve maximum precipitation of the proteins. The precipitates were sedimented by centrifugation at 15,000 x g at 4°C for 20 min and reconstituted in a minimum volume of 0.05 M Tris buffer (pH 8.0). The

supernatant and reconstituted precipitate fractions were analyzed separately for esterase activity and protein.

Substrate specificity of esterase produced by *B. safensis* strain

The effect of different *p*-nitrophenyl acyl esters/ substrates on the partially purified esterase was evaluated by using *p*-NPA, *p*-NPL, *p*-NPB, *p*-NPM or *p*-NPP at 10 mM concentration in reaction mixture containing 0.05 M Tris buffer (pH 8.5) and cell-free esterase and incubating at 37°C for 10 min (under shaking). The absorbance readings were taken at 410 nm after stopping the reaction by chilling at -40°C.

Effect of substrate (*p*-NPA) concentration

To optimize the concentration of selected substrate (*p*-NPA), the partially purified esterase was used at 4, 6, 8, 10, 12, 15 and 20 mM in 0.05 M Tris buffer (pH 8.5) at 37°C for 10 min (under shaking) and activity was determined thereof.

Determination of K_m and V_{max}

The kinetic parameters such as K_m and V_{max} of the partially purified esterase were determined by plotting the reciprocal of the reaction velocity (1/V) against the reciprocal of the substrate (*p*-NPA) concentration (1/S, 1/4 to 1/20 mM) using Line weaver-Burk plot. The reaction was performed by incubating the reaction mixture containing 0.05 M Tris buffer (pH 8.0), *p*-NPA (different concentrations) and 100 µl of cell-free esterase, at 40°C for 15 min under shaking conditions.

Molecular weight determination and establishment of purity of esterase by PAGE

The native as well as subunit structure/ *Mr* were determined for purified esterase of selected bacterial isolate by using a Gel Doc system. The acetone precipitated protein was run under denaturing (SDS-PAGE) and non-denaturing (native-PAGE) conditions using a 12 % acrylamide gel [16]. For measurement of molecular mass of protein, commercial molecular mass standard proteins were used.

Results and discussion

Isolation and screening of esterase producing bacteria

The ear wax/sebum samples collected from different individuals were processed for isolation of lipolytic bacterial strains using qualitative and quantitative screening. Twenty one bacterial isolates from sebum samples of five individuals produced clear zones around colonies on Tributyrin (1.0 %; v/v) agar plates. Out of 21 bacterial isolates, 10 bacterial isolates were subjected to qualitative (primary) and quantitative (secondary) screening (Fig. 1). The bacterial isolates namely 'R5', 'S8', 'J7' and 'S1' showed hydrolyzing efficiency between 100 to 140 % (Table 1). Among all the selected bacterial isolates, the bacterial isolate 'S8' showed a relatively higher esterase ac-

Fig. 1 (a)

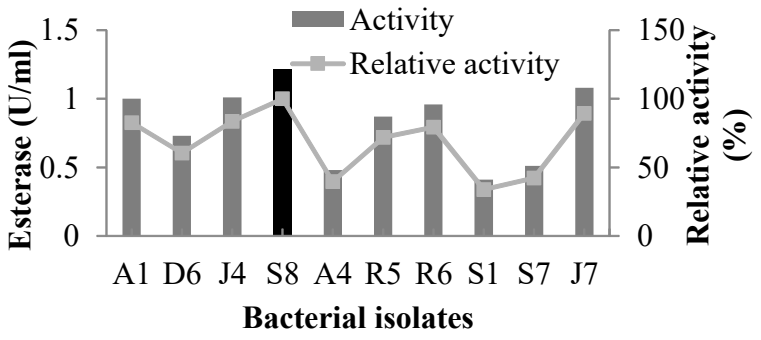

Fig. 1 (b)

Fig. 1a. Tributyrin agar plate showing zone of hydrolysis around the bacterial colonies (qualitative screening).
Fig. 1b. Extracellular esterase activity of selected bacterial isolates.

Table 1. Biochemical characteristics of bacterial isolate 'S8'

Biochemical tests	Isolate 'S8'	*B. pumilus*	*Bacillus safensis*
Catalase	+ve	+ve	+ve
Malonate utilization	-ve	-ve	-ve
Voges Proskauer's	+ve	+ve	+ve
Citrate utilization	-ve	+ve	-ve
ONPG	+ve	+ve	+ve
Arginine utilization	-ve	-ve	-ve
Esculin hydrolysis	+ve	+ve	+ve
Carbohydrate fermentation			
Sucrose	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve
Glucose	+ve	+ve	+ve
D-arabinose	-ve	-ve	-ve
Trehalose	+ve	+ve	+ve
Maltose	-ve	-ve	+ve
Raffinose	+ve	+ve	-ve
Inositol	-ve	-ve	+ve
Methyl-alpha-D-glucopyranoside	-ve	-ve	+ve
Melibiose	+ve	-ve	+ve
Inulin	+ve	-ve	-ve
Salicin	-ve	+ve	+ve
Cellobiose	-ve	+ve	+ve

tivity (1.21 U/ml). Thus the bacterial isolate 'S8' that produced maximum esterase activity in MB broth was selected for further studies.

Characterization and molecular identification of bacterial isolate 'S8'

The colonies of esterase producing bacterial strain 'S8' were creamish in colour and elevated with margins. The strain 'S8' was found Gram's positive, rod shaped and endospore forming bacteria. The 16S rRNA gene sequencing clearly demonstrated that isolate 'S8' was a member of the genus '*Bacillus*' (Fig. 2). The sequence analysis and BLAST analysis revealed that isolate 'S8' had 96 % identity with *Bacillus pumilus* and *Bacillus safensis* strain. The phylogenetic tree constructed using 16S rRNA gene sequences of other related members of *Bacillus* sp. revealed that isolate 'S8' (sample) formed a close cluster with *B. pumilus* and *Bacillus safensis* strains (Fig. 3). Most of the biochemical characteristics of isolate 'S8' were similar to *Bacillus safensis* than *Bacillus pumilus* (Table 1). On the basis of morphological characteristics, biochemical characteristics and 16S rRNA gene sequence analysis, isolate 'S8' was identified as *B. safensis* strain.

Enhancement of esterase production by optimizing physico-chemical parameters

Various physico-chemical parameters were optimized in order to increase esterase production by *B. safensis* strain.

Effect of incubation time on esterase production by *B. safensis* strain

The optimal incubation time for production of extracellular esterase by *B. safensis* strain was found to be 36 h at 37^0C in MB broth that corresponded to a maximum enzyme activity of 1.51 U/ml. Any further increase in incubation time did not show any improvement in enzyme activity and it rather decreased gradually (Fig. 4). Thus the production time of 36 h at 37^0C was considered optimum for extracellular esterase production by

GCGTCCGCTGGCGAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCG
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT
AAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCC
TGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGAC
ATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGC
TACACACGTGCTACAATGGACAGAACAAAGGGCTGCAAGACCGCAAGGTTTAGCCA
ATCCCATAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCT
GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGT
ACACACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAGTCGGTGAGGTAACC
TTTATGGAGCCAGCCGCCGAAGGTGGGGCAGATGATTGGGGGTGAAGTCTCGGGGG
GGGCCCCATATATACCGTCATCTCAGGCGGAGTGCTTATGCGTTAGCTGCAGCACTA
AGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGG
GTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGA
GAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTG
GAATTCCACTCTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCAATGACCCTCCCCGGT
TGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCA
ATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTA
GCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCGAGCAGTTACTCTCGCACTTGTT
CTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGC
TCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCT
GGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGT
CGCCTTGGTGAGCCATTACCCCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAA
GTGACAGCCGAAACCGTCTTTCATCCTTGAACCATGCGGTTCAAGGAACTATCCGGT
ATTAGCTCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTA
CTCACCCGTCCGCCGCTAACATCCGGGAGCAAGCTCCCTTCTGTCCGCTCGACTTGC
ATGTATTAGCACGCCGCCAGCGTTCGTCTGAGAGGGGAACTTTTCTCTTTCTTTTT

Fig. 2. Consensus sequence of bacterial isolate 'S8'

B. pumilus strain. Maximum production of feruloyl esterase from *Aspergillus niger* was reported after 72 h of fermentation [19]. The highest esterase production from marine *Vibrio fischeri* was observed after 72 h of incubation at 30ºC [22].

Effect of carbon source and its concentration

Carbon is a main component of cells and here oils were used as a carbon source or inducer for esterase production. MB broth was separately supplemented with each of the selected oil and incubated for 36 h at 37^{0}C under shaking (160 rpm). *B. safensis* strain produced maximum enzyme in MB broth in the presence of olive oil or coconut oil with esterase activity of 1.84 and 1.82 U/ml, respectively and these values were relatively higher than other oils (Fig. 5). However, olive oil is being expensive than coconut oil, therefore, coconut oil was considered for supplementation in the MB broth for further optimization studies for extracellular esterase production by *B. safensis* strain. Earlier, castor oil was reported as the best lipid source for the esterase production by *Bacillus circulans* [8]. In contrast, *V. fischeri* utilized Tween 20 and tributyrin as carbon source and energy material to produce esterase as oils were not utilized by this strain [22]. The coconut oil concentration had a strong influence on the production of esterase by *B. safensis* strain, as given in (Fig. 6). with an increase in coconut oil concentration up to 1.75 %, there was an increase in esterase activity and then slowly decrease in the

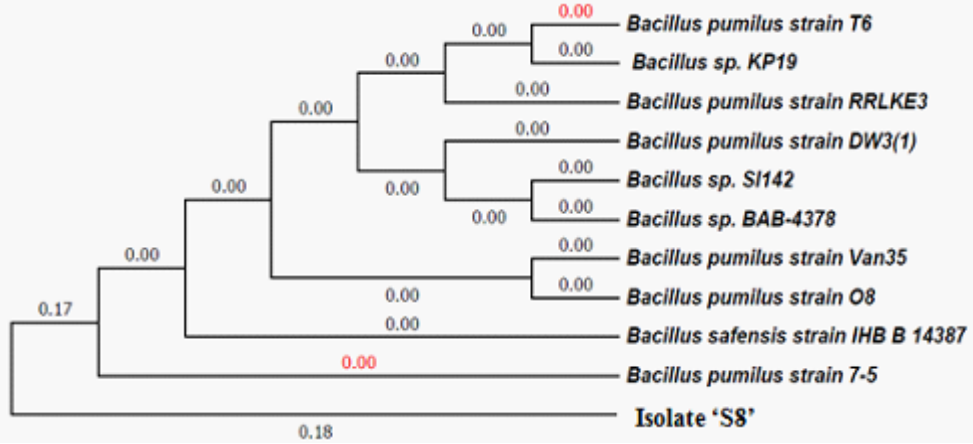

Fig. 3. Phylogenetic tree showing relationship between Bacillus species and bacterial isolate 'S8'.

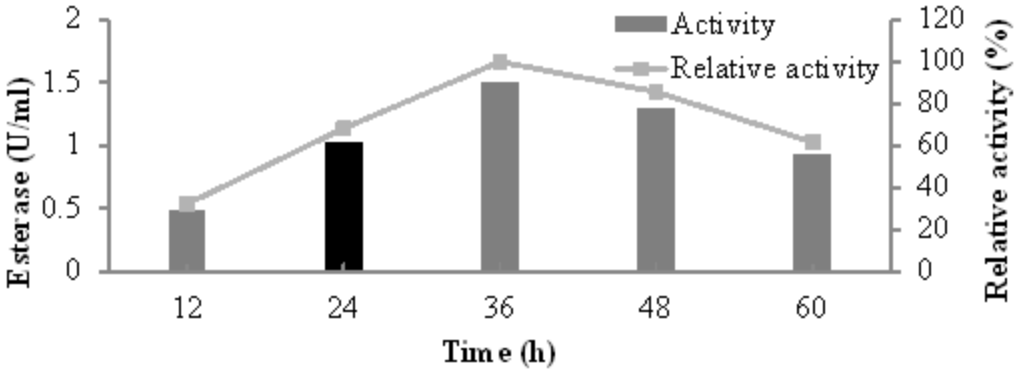

Fig. 4. Effect of incubation time on production of esterase by *B. safensis* strain.

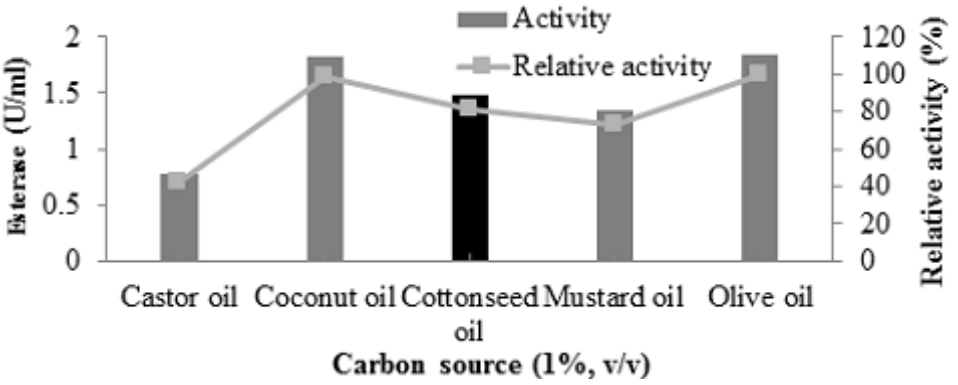

Fig. 5. Effect of oil/ carbon source on production of esterase in MB broth by B. safensis strain.

esterase activity.

Effect of nitrogen source and its concentration

Organic nitrogen sources play an important role in the synthesis of enzymes because they provide many cell growth factors and amino acids, which are required for cell metabolism and enzyme synthesis. The maximum esterase production (2.82 U/ml) was observed when gelatin was used as a nitrogen source that was higher than the yeast extract which being used as nitrogen source before this experiment (Fig. 7). Maximum esterase activity (3.1 U/ml) was observed when 0.6 % (w/v) of gelatin was supplemented in the MB broth (Fig. 8). In a previous study, maximum amount of esterase produced was recorded in ammonium hydrogen carbonate supplemented medium. But the lowest amount of enzyme production was

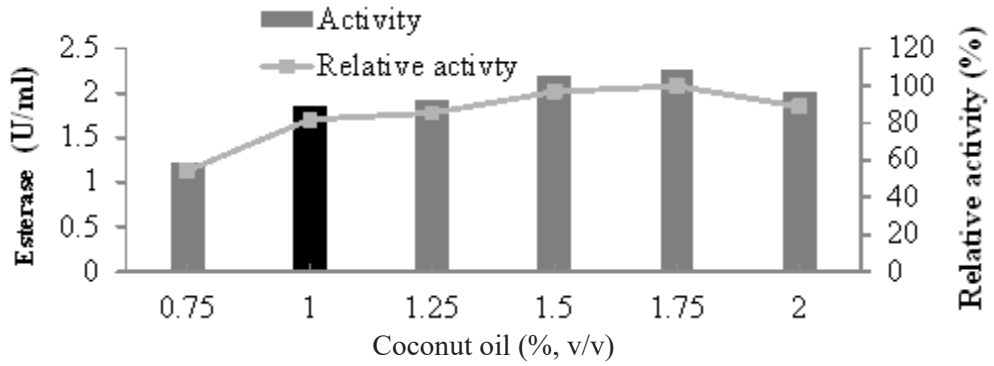

Fig. 6. Effect of concentration of coconut oil on production of esterase in MB broth by *B. safensis* strain.

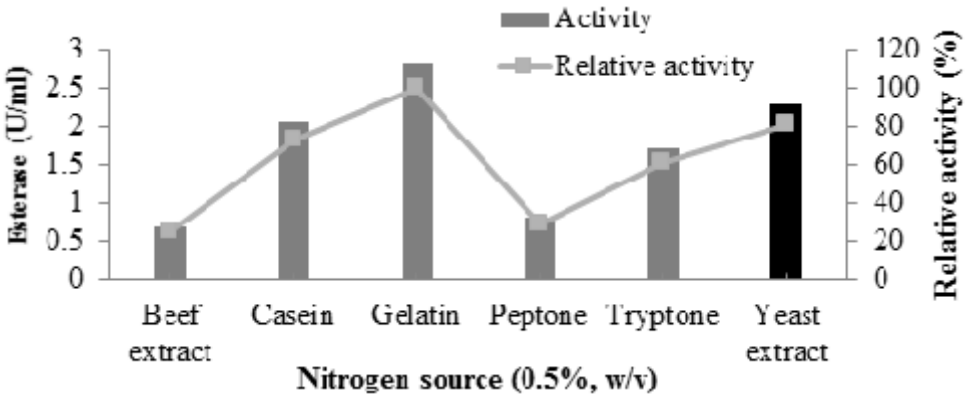

Fig. 7. Effect of nitrogen source on production of esterase in MB broth by *B. safensis* strain

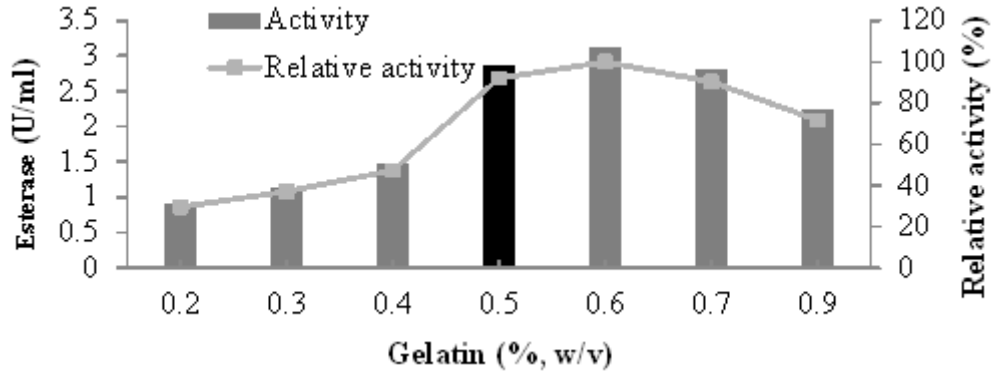

Fig. 8. Effect of concentration of gelatin on production of esterase in MB broth by *B. safensis* strain.

observed in tryptone supplemented medium [8].

Optimization of production temperature

Most favourable temperature for esterase production in MB broth by *B. pumilus* strain was found to be 37^{0}C (3.13 Uml). At 25^{0}C, esterase production was low but as the temperature rose from 25 to 37^{0}C, there was a corresponding increase in esterase production (Fig. 9). This indicated the mesophilic nature of the *B. safensis* strain. This observation was quite likely as the bacterium was isolated from human ear wax, a natural habitat being maintained by the host. Similarly, the optimum temperature for esterase production by *Bacillus* sp. was found 37°C [21]. It was observed that 30ºC was generally more favorable temperature for esterase production from marine *Vibrio fischeri* [22].

Optimization of inoculum size

The MB broth inoculated with 2 % (v/v) inoculum resulted in maximum esterase production (3.10 U/ml) after 36 h incubation at 37°C (Fig. 10). Thus use of inoculum at 2 % (v/v) was considered optimum for good esterase production by *B. safensis* strain. The optimal level of inoculum concentration was between 3.0 and 3.5 % for esterase production by the yeast *Pseudozyma* sp. NII 08165 [1].

Selection of broth for optimal esterase production

Among different broths used for esterase production by *B. safensis* strain, maximum esterase production of 3.14 U/ml was noticed in the originally used MB broth (control) followed by Muller- Hinton broth. Moreover, least esterase activity was recorded in the Luria Bertani broth (Fig. 11).

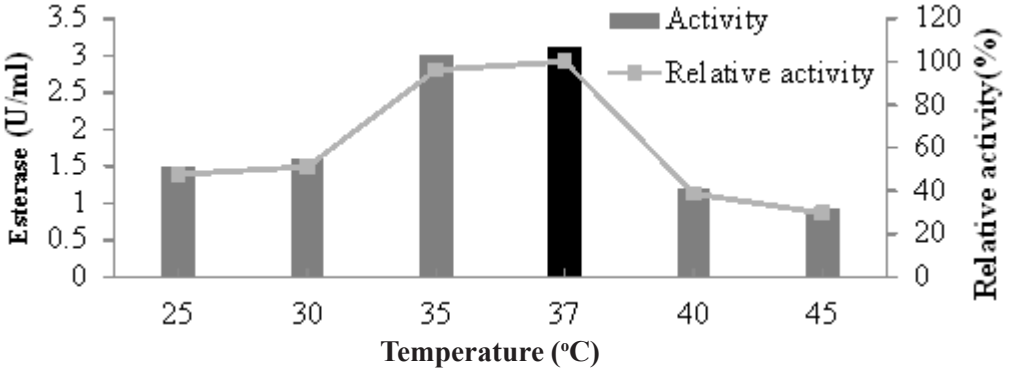

Fig. 9. Effect of temperature on production of esterase by *B. safensis* strain.

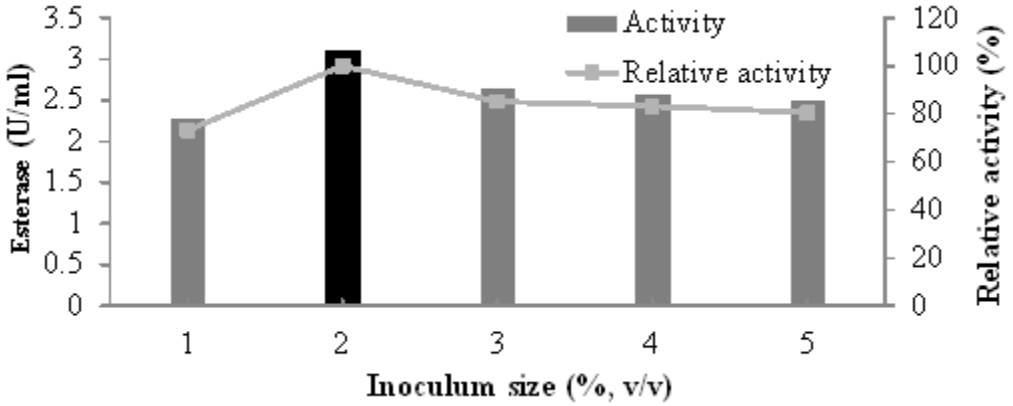

Fig. 10. Effect of inoculum size on production of esterase by *B. safensis* strain.

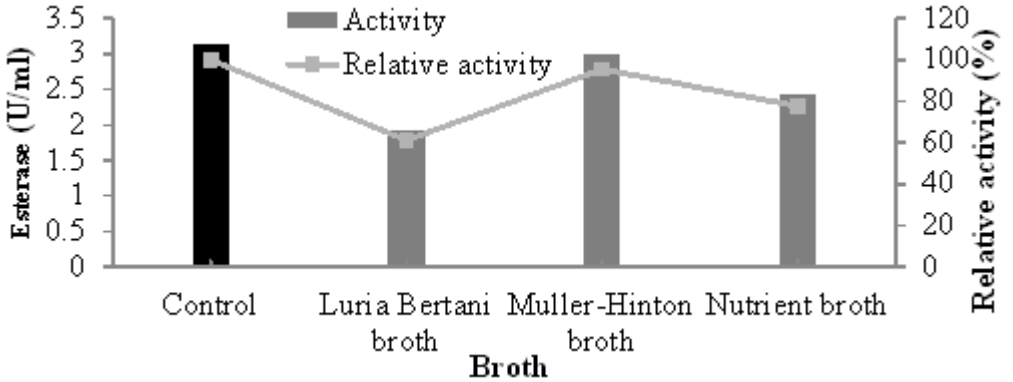

Fig. 11. Selection of production broth for optimal esterase production by *B. safensis* strain.

Substrate specificity of esterase of *B. safensis* strain

The bacterial esterase was more efficient to hydrolyze *p*-NPA (C: 8) than *p*-NPP having a longer C-length (C: 16). Moreover, the esterase activity recorded in the presence of *p*-NPA was approximately 36 % higher than *p*-NPP. The enzyme showed little activity in the presence of an aromatic ester (*p*-NPB). In contrast, *L. brevis* NJ13 showed highest activity towards *p*-NPB and 59 % lower towards *p*-NPA [11]. Esterase and lipase differ from each other on the basis of the physicochemical nature of the substrate and length of the substrate (fatty acid) carbon chain [7]. An esterase prefers a water-soluble substrate and generally displays activity toward triglycerides composed of short carbon-chain fatty acids. On the contrary, a lipase prefers water-insoluble substrates composed of long carbon-chain fatty acids [2].

In the present study, esterase produced by *B. safensis* strain hydrolyzed the substrates in order *p*-NPA> *p*-NPP> *p*-NPL> *p*-NPM> *p*-NPB (Fig. 12). Esterase produced by a ruminal bacterium *But-yrivibrio fibrisolvens* degraded substrates at a rate in the order 1-naphthyl acetate > 1-naphthyl butyrate > 1-naphthyl propionate but did not degrade 1-naphthyl palmitate or 1-naphthyl phosphate [17]. The purified esterase from *Thermus* sp. NCCB 100425^T had maximal relative activity towards *p*-NPA and *p*-NPB, respectively, whereas it could slightly cleavage ester bonds of *p*-NPL and *p*-NPP [26].

Effect of substrate (*p*-NPA) concentration

The maximum esterase activity (4.81 U/ml, Fig. 13) was observed when *p*-NPA of 10 mM concentration was used in the assay mixture containing 0.05 M Tris buffer (pH 8.5) and cell-free broth/crude esterase, which was incubated for 10 min at 37°C under shaking. A further increase in substrate concentration resulted in decline in the esterase activity.

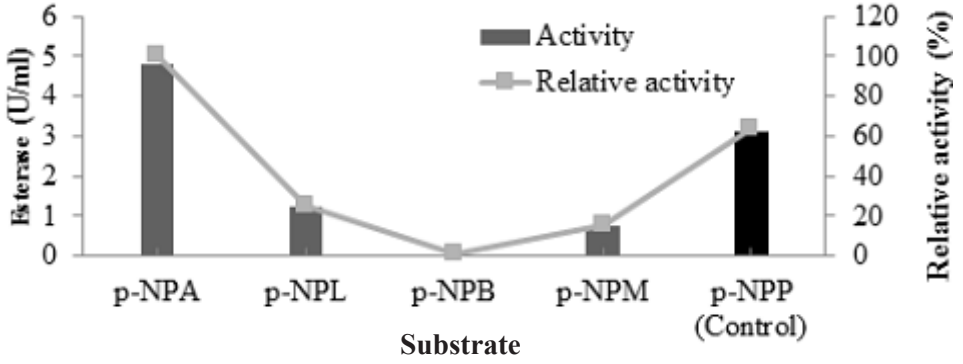

Fig. 12. Effect of different p-nitrophenyl acyl ester substrates on the activity of esterase of *B. safensis* strain.

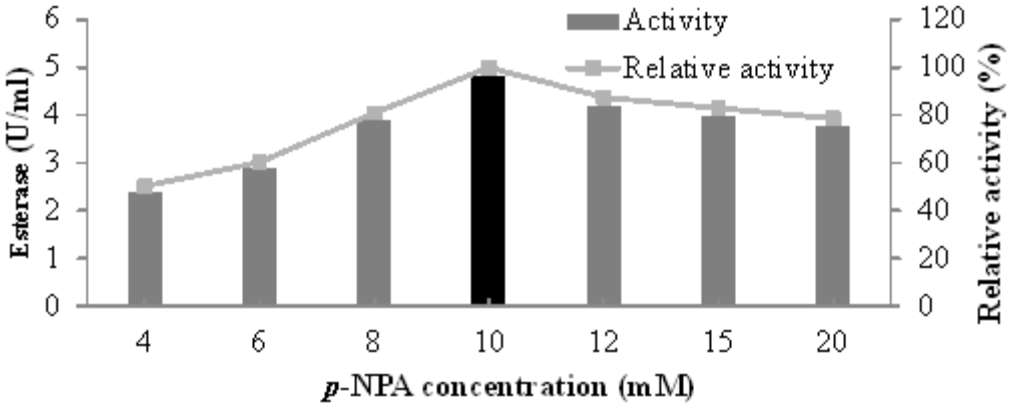

Fig. 13. Effect of different concentrations of p-NPA on the activity of esterase of *B. safensis* strain.

Determination of K_m and V_{max}

The K_m and V_{max} values of the esterase by *B. safensis* strain in the presence of *p*-NPA as a substrate were found to be 0.127 mM and 8.82 U/ml/min, respectively (Fig. 14). A relatively lower K_m value indicated higher affinity of the esterase of *B. safensis* strain towards the substrate *p*-NPA. In a previous study, the K_m 0.91 mM and V_{max} 213 Um/min/mg were recorded for an esterase of *Bacillus* sp. [21]. Esterase produced by a ruminal bacterium identified as *Butyrivibrio fibrisolvens* showed a K_m of 7.6 x10^{-4} on naphthyl acetate [17].

Purification and molecular weight determination of esterase of *B. safensis* strain

The maximum esterase activity (16.3 U/ml) and specific activity (203.6 U/mg) was observed at 20-40 % saturation. The esterase of *B. safensis* was purified up to 42.8 fold simply by acetone precipitation. The analysis of the PAGE under denaturing and non-denaturing conditions revealed a single band of 47 kDa (Fig. 15(a)) and 4 49 kDa (Fig. 15b), respectively. These results suggested that extracellular esterase fractionated by acetone was successfully purified to homogeneity and the purified esterase protein appeared to be a heptamer protein of 49 kDa comprising seven units of 47 kDa. Acetone was used at different saturations to purify lipase from *Rhizopus japonicas* [25]. They reported that the molecular mass of metal insensitive lipase isolated from *Rhizopus japonicas* was 30 kDa. The molecular weight of acetylesterase by *Bacillus* sp. was 62 kDa [21].

The purified esterase from the yeast *Pseudozyma* sp. NII 08165 showed a single band of molecular weight 175 kDa on SDS-PAGE [1]. An esterase puriûed from the golden grey mullet viscera was found to be a monomer having molecular mass of about 55 kDa, as determined by SDS-PAGE analysis [23].

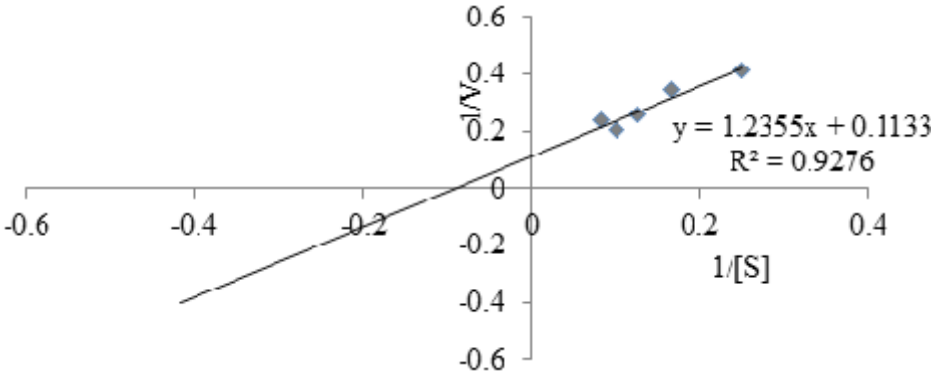

Fig. 14. Lineweaver-Burk plot for determination of K_m and V_{max} values of esterase of *B. safensis* strain

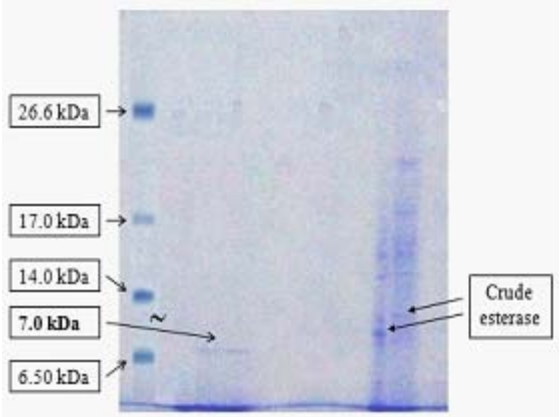

Fig. 15a. SDS-PAGE of partially purified esterase of *B. safensis* strain. From left to right, Lane 1. Reference protein markers of 6.5-26 kDa, lane 2 and 3. Purified esterase

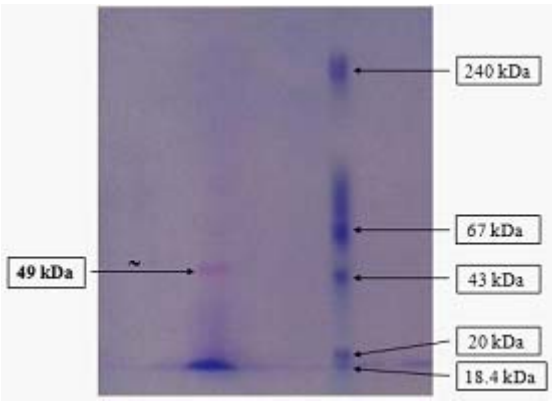

Fig. 15b. Native-PAGE of partially purified esterase of *B. safensis* strain. From left to right the Lane 1. Precipitated esterase Lane 2. Bangalore Genei protein marker

Conclusion

An alkaliphilic extracellular esterase of *B. safensis* strain isolated from human ear wax/ sebum possessed high specificity/ affinity towards *p*-NPA which is a relatively short chain ester. The esterase production by *B. safensis* strain increased by ~208% by consecutive optimization of physico-chemical parameters. The esterase was efficiently precipitated with acetone and it was found to possess a mass of 49 kDa comprising seven units of 4 7 kDa as analyzed by native PAGE and SDS-PAGE, respectively. The multimeric nature of the esterase of *B. safensis* strain appears to be a unique property of the esterase. The K_m and V_{max} values of the esterase by *B. safensis* in the presence of *p*-NPA were found to be 0.127 mM and 8.82 U/ml/min, respectively The esterase of *B. safensis* strain may be used in ester synthesis so that its application may be explored in achieving biocatalysis and biotransformation of short chain fatty acids into useful esters, eco-friendly activities such as decolorization of dyes, degradation of plastics, in production of optically active compounds, or in food industry.

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