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# Molecular Cloning and Characterization of a Lipase from an Indigenous *Bacillus pumilus*

K. Morabbi Heravi,[2] B. Yakhchali,[1,*] F. Eftekhar,[2] B. Vafadar-Isfahani,[3] H. Habib Ghomi,[1] and H. Ahmadi-Danesh[1]

[1]*Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology, Pajouhesh Blvd., Tehran-Karaj Highway, Tehran, Isalmic Republic of Iran*
[2]*Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Chamran Highway, Evin, Tehran, Isalmic Republic of Iran*
[3]*The JohnVan Geest Cancer Research Centre, School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS*

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## Abstract

Cloning and sequencing of a lipase gene from an indigenous *Bacillus pumilus*, strain F3, revealed an open-reading frame of 648 nucleotides predicted to encode a protein of 215 residues. Sequence analysis showed that F3 lipase contained a signal peptide composed of 34 amino acids with an H domain of 18 residues. A tat-like motif was found in the signal peptide similar to some other *Bacillus pumilus* lipases. High similarity was also observed between the protein sequences of the lipases from *B. pumilus* strains and F3 lipase especially near the catalytic triad, oxyanion hole and tentative lid. Expression of the lipase gene with its native signal peptide in *Escherichia coli* BL21 (DE3) led to accumulation of lipase as inclusion bodies in cytoplasm due to high hydrophobicity of the protein. Despite inclusion body formation, slight lipolytic activity was detected in periplasm as well as soluble cytoplasmic protein fractions which could show the presence of the recombinant lipase in the periplasmic space of host cell. This may indicate the processing of the lipase by its native signal peptide, although incapable to translocate the enzyme into the *Escherichia coli* periplasm completely.

**Keywords:** *Bacillus pumilus*; *Escherichia coli* BL21; Lipase; Cloning

## Introduction

Lipases (Triacylglycerol acylhydrolases; EC 3.1.1.3) catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids at the interface between water and oil. These enzymes are also capable of catalyzing the reverse reaction in micro-aqueous conditions [15,16,36]. Numerous microorganisms including fungi and bacterial genera such as *Pseudomonas*, *Bacillus*, and *Burkholderia* produce lipases [16,29,35]. Bacterial lipases are categorized into eight families and lipases from Bacilli belong to I.4 and I.5 subfamilies [1]. Lipases from *B. pumilus*, *B. subtilis* and *B. licheniformis* belonging to subfamily I.4 are smaller than other

* Corresponding author, Tel.: +98(21)44580353, Fax: +98(21)44580399, E-mail: bahar@nigeb.ac.ir

subfamilies and lack the lid structure [1, 40]. Some lipases such as the lipase from *B. pumilus* B26 do not contain $Ca^{2+}$ binding motif near the catalytic histidine site and their activity is $Ca^{2+}$ independent. Features such as $Ca^{2+}$ independent thermostability, enantioselectivity, alkalotolerance, independency of cofactors and not catalyzing side reactions make these enzymes useful in industry particularly in laundry detergents [16,17,20,29, 35,36].

Determination of the molecular properties of lipases has been mostly carried out by gene cloning in heterologous hosts such as *E. coli* [9,19,25,30,33]. Most studies have been performed on lipases from *B. subtilis,* and *B. pumilus* lipases have not been extensively investigated [13,14,25,40]. In our study, the lipase gene from a soil isolate of *B. pumilus* was cloned and expressed in *E. coli*. The lipase gene was then sequenced and based on the information obtained the structure and molecular properties of the enzyme were predicted and compared to lipases from other *B. pumilus* strains.

## Materials and Methods

### *Strains, Plasmids, and Mdia*

An alkalotolerant *Bacillus pumilus* F3, a soil isolate, was used as the lipase source for cloning studies [12]. Plasmid pTZ57R/T (Ins T/A clone, PCR product cloning kit, #k1214, Fermentas) was used as the cloning vector for cloning of the lipase gene in *E. coli* TG1 strain and pET-26b(+) (Novagen) was used for enzyme expression in *E. coli* BL21 (DE3) (Novagen). Lauria Bertani (LB) medium was used for growing *Bacillus pumilus* F3 at 30°C and *E. coli* strains at 37°C. LB agar containing 100 µg/ml ampicillin, 0.5 mM IPTG and 20 µg/ml X-Gal was used to screen the recombinant clones harboring pTZ57R/T plasmids. *E. coli* BL21 harboring plasmid pET-26b (+) was screened on LB medium containing 60 µg/ml kanamycin [34].

### *Sequencing of Lipase Gene*

Plasmid and genomic DNA extractions were performed by phenol/chloroform as described previously [34]. To amplify the lipase gene, primer oligonucleotides Lip B26F (5'- GAG TCG AAT CAT ATG AAT AAG G -3') and Lip B26R (5'- TGG ATC CAT TTT TTA ATT CGT ATT CTG -3') were designed by aligning DNA sequences of lipases from *B. pumilus* strains submitted to National Center for Biotechnology Information (NCBI) and were constructed by Gene Fanavaran Company (Tehran, Iran). Amplification of the lipase gene was carried out in a 50 µl reaction mixture containing 5 µl of 10 x PCR buffer, 2 µl dNTP mixture (10 mM), 1.5 µl $MgCl_2$ (50 mM), 2 µl of each nucleotide primer (10 pmol/µl), 1 µl of DNA (5-10 ng) and 0.6 µl Taq DNA polymerase (5 U/µl) in a thermocycler (Techne Flexigene, Model FFG05TUD, Minneapolis, MN, USA) using the following program. Five cycles of 45" at 94°C, 1' at 43°C, and 1' at 72°C followed by 25 cycles of 45" at 94°C, 1' at 53°C, and 1' at 72°C. All PCR chemicals were purchased from Fermentas. Universal primers M13/pUC were employed to confirm the presence of the lipase gene in recombinant clones using the following program: 30 cycles each 45" at 94°C, 1' at 51°C, and 1' at 72°C. DNA was sequenced using the dideoxy termination method with ABI automated sequencer at the Research Center for Gastroenterology and Liver Diseases (RCGLD), Taleghani Hospital (Tehran, Iran).

### *Expression of Recombinant Lipase in* E. coli *BL21*

Using plasmid pYM122 as DNA template, the lipase gene was amplified by F. EXP (5'- AAT AAG GG CAT ATG AAA GTG ATT CG -3') and Lip B26F oligonucleotides primers. Amplification was performed using 30 cycles of 45" at 94°C, 1' at 55°C, and 1' at 72°C. The amplified fragment was cloned into pTZ57R/T which was then named pYM123. The lipase gene was subcloned into plasmid pET-26b(+) by double digestion of pET-26b(+) and pYM123 using *Bam*HI and *Nde*I restriction enzymes (Fermentas). Subsequently, pET-26b(+) containing lipase gene (pYM124) was transferred into BL21 strain of *E. coli* K12 according to the standard protocol [34]. Lipase expression was performed by growing of the transformant in a 500 ml Erlenmeyer flask containing 100 ml LB medium containing kanamycin (60 µg/ml) at 30°C with agitation at 100 rpm. The culture was induced by 0.2 mM IPTG after 2 h of incubation ($OD_{600}$ 0.7) and harvested 4 h and 20 h after induction. The supernatant was used for electrophoresis and enzyme assay studies. The pellets were subjected to osmotic shock according to the method of Rastgar Jazii *et al.* with a slight modification [31]. Bacterial culture was centrifuged at 4000 rpm (J-6, Beckman) for 10 minutes at 4°C followed by suspension of pellet in 15 ml TES buffer (0.2 M Tris-HCl pH 8.0, 0.5 mM EDTA and 0.5 M sucrose) per liter of the original culture and were placed on ice for 30 minutes. Osmotic shock was performed by adding 22.5 ml cold water and incubating on ice for 30 min before centrifugation at 8,000 rpm (Heraeus D-37520 Osterode, Germany) for 5 min to collect the supernatant containing the periplasmic fraction. The sphaeroblast

pellet was suspended in 0.05 M Tris-HCl, pH 8 followed by sonication for 10 min (70% amplitude and 0.5 cycle on ice, Hielscher, UP200S, Germany) and centrifuged at 13,000 rpm for 5 min. The supernatant contained soluble cytoplasmic proteins while the pellet contained inclusion bodies.

Expression analysis and determination of the molecular weight of the recombinant protein was carried out on a 13% SDS-polyacrylamide gel according to Laemmli method [23].

### *Lipase Assay*

Lipolytic activity was determined spectrophotometrically using 1.5 mM *p*-nitrophenyl palmitate (Sigma) as described by Cho *et el.* 2000 [9]. One unit of lipase was defined as the amount of enzyme liberating 1 µmol of *p*-nitrophenol per minute.

### *Analysis of Lipase*

Amino acid sequence of the F3 lipase was deducted from its DNA sequence by EditSeq 3.82d3 in DNASTAR Lasergene software [8]. SignalP 3.0 [4] and TMHMM 2.0 [22] were used for signal peptide and H domain prediction, respectively. The protein sequences of F3 lipase and other known lipase genes were aligned using Bioedit 7.0.5.3 [38] and CLUSTAL X 1.81 [37]. Molecular characteristics of lipases such as molecular weight, isoelectric point, charge at pH 7 and the number of different residues were determined from the protein sequences using the EditSeq program (Table 2). The secondary structure of the first 100 nucleotides of transcripted RNA from pYM124 (pET-26b(+) containing lipase gene) was predicted by RNA 2 prediction software [6,7].

### *In-Gel Tryptic Digestion*

The excised SDS gel slice was washed with water and 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate until residual SDS and Coomassie blue dye were completely removed. The destained gel slices were shrunk by dehydration with 100% acetonitrile (ACN) and rehydrated in 50 mM ammonium bicarbonate. This was followed by dehydration with 100% acetonitrile and dried in a vacuum centrifuge. The gel slices were then digested overnight with trypsin at 37°C by adding 33.2 µl of ammonium bicarbonate, 15.2 µl of water and 1.4 µl of mass spectrometry grade trypsin gold (Promega, Southampton, UK). The enzyme reaction was terminated by addition of 0.1% trifluoroacetic acid (TFA) in water. Peptides extraction was carried out by addition of 100 µl 0.1% TFA:CAN (1:1) solution to the gel band and sonication which was repeated twice. This was followed by $C_{18}$ ZipTip reversed-phase chromatography of the supernatant according to the manufacturer's instructions (Milipore, Watford, UK). Following $C_{18}$ ZipTip clean-up, 1 µl of the eluted sample was spotted on the matrix-assisted laser desorption/ionization (MALDI) target plate with 1 µl of matrix solution of 10 mg α-cyano-4-hydroxycinnamic acid (CHCA) in 1 ml of 50% ACN/0.1%TFA and allowed to air dry at room temperature.

### *Mass Spectrometry and Protein Identification*

For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis, an Ultraflex MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen Germany) was used. The reflectron positive mode was used for analysis of tryptic peptide gel sample in the mass-to-charge (*m/z*) of 700-4000; peptide calibration mix 4 (Laser Bio Labs, Cedex France) was used for calibration of the instrument which was based on monoisotopic masses and contained Bradykinin fragment 1-5 *m/z* 573.31, Angiotensin *m/z* 1046.54, Neurotensin *m/z* 1672.91, ACTH clip (18-39) *m/z* 2465.19 and Insulin B-chain oxidized *m/z* 3494.65. To prepare the calibration mixture for spotting on the plate, equal volumes of the appropriate calibration solution was mixed with the relevant matrix (10 mg/ml CHCA in 50%ACN/50/ %TFA). Subsequently, 1µl of this solution was spotted manually on the plate. Obtained spectra were elaborated with the FlexAnalysis® and BioTools® software (Bruker Daltonics) and the resulting mass list were subjected to MASCOT (www.matrixscience.com) on a local server.

## Results and Discussion

### *Cloning and Sequencing of the Lipase Gene*

Amplification of *Bacillus pumilus* F3 lipase gene using Lip B26F and Lip B26R primers yielded a fragment with an approximate size of 680 bp. The fragment was cut out of the gel, purified and cloned into pTZ57R/T vector followed by transformation of *E. coli* TG-1 host. After verification of the presence of lipase gene, the recombinant plasmid (pYM122) was sequenced using universal primer M13/pUC forward. The sequencing results revealed a 648 bp ORF encoding the lipase precursor of *Bacillus pumilus* F3 which was then compared with lipases from other *Bacillus* sp. using Blast (blastn algorithm). The results showed 91% similarity to *Bacillus pumilus DBRL-191* and *B. pumilus*

**Table 1.** Lipases originating from *B. pumilus* strains. Tat pathway motif and its similar sequences are shown in boxes. N and C charges demonstrate the net charge of amino and carboxyl domains of the signal peptide. Transmembrane helices (H domains) predicted by TMHMM 2.0 [22] are highlighted

| Strain | Amino acids | Sequence | N charge | C charge |
|---|---|---|---|---|
| F3 | 34 | MKVIRFKKRSLQILVVLALVMGSMAFIQPKEIRA | +5 | +1 |
| YZ02 | 34 | MKVMFVKKRSLQILIALALVIGSMAFIQPKEVKA | +4 | +1 |
| DSM 5776 | 34 | MKVIFVKKRSLQILIALALVIGSMAFIQPKEAKA | +4 | +1 |
| DBRL-191 | 34 | MKVILFKKRSLQILVALALVIGSMAFIQPKEVKA | +4 | +1 |
| XJU-13 | 31 | IRFKKKSLQILVALALVIGSMAFIQPKEAKA | +4 | +1 |
| B26 | 34 | MKVIRFKKKSLQILVALALVLGSIAFIQPKEAKA | +5 | +1 |
| LipA | 31 | MKFVKRRIIALVTILMLSVTSLFALQPSAKA | +4 | +1 |

**Table 2.** Comparison of the properties of the F3 lipase with lipases from other *B. pumilus* strains

| Strain | Mature protein | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Residues | pI[a] | $M_w$[b] | pH 7[c] | +[d] | -[e] | H[f] | P[g] | Ref |
| F3 | 181 | 9.623 | 19253.90 | 6.737 | 18 | 12 | 66 | 50 | This study |
| YZ02 | 181 | 9.469 | 19303.90 | 5.737 | 18 | 13 | 62 | 52 | accession number DQ339137 |
| DSM 5776 | 181 | 9.469 | 19334.00 | 5.737 | 18 | 13 | 62 | 53 | [27] |
| DBRL-191 | 181 | 9.440 | 19199.80 | 5.736 | 18 | 13 | 62 | 52 | [30] |
| XJU-13 | 179 | 9.496 | 19010.60 | 5.738 | 17 | 12 | 65 | 49 | accession number EF434173 |
| B26 | 181 | 9.496 | 19225.80 | 5.738 | 17 | 12 | 65 | 51 | [20] |
| LipA | 181 | 9.469 | 19334.00 | 5.737 | 18 | 13 | 62 | 53 | [11] |

**a:** Isoelectric point; **b:** molecular weight (daltons); **c:** charge at pH 7.0, d, strongly basic amino acids (K,R); **e:** strongly acidic amino acids (D,E); **f:** hydrophobic amino acids (A,I,L,F,W,V); and **g:** polar amino acids (N,C,Q,S,T,Y).

B26 lipases, 90% similarity to *B. pumilus* YZ02 and *B. pumilus* XJU-13, and 69% homology to LipA from *B. subtilis* 168 (NCBI accession number EF093106).

### *In Silico Analysis of Protein Sequence*

Sequence analysis of lipase F3 protein revealed 215 residues (Genbank Accession number ABK80759). The results showed 94% similarity to *Bacillus pumilus* B26 and *B. pumilus* XJU-13, 93% homology to *B. pumilus* DBRL-191 lipases, 92% similarity to *B. pumilus* YZ02, and 73% homology to LipA from *B. subtilis* 168 (Fig. 1).

Further analyses showed a signal peptide of 34 amino acid residues harboring an H domain with 18 amino acids (Table 1). Presence of KR residues in the N-terminal portion of the signal peptide may present a Tat secretion pathway. However, amino acid Q12 located at +3 position downstream of KR, presents a hydrophilic residue unlike the Tat motif (KRX or RRX where indicates hydrophobic residues) [18, 39]. Our investigation showed that the signal peptide of the F3 lipase contained motifs in the N-terminus of the H domain similar to the Tat motif found in LipA, YZ02, DSM 5776 and DBRL-191 lipases (KRX or RRX where indicates hydrophobic residues). This shows an evolutional similarity despite the replacement of hydrophobic amino acids by hydrophilic residues. Most of the *Bacillus* lipases are secreted through the Sec pathway [17]. Other lipases originated from *Staphylococci* and *Pseudomonas* sp use different secretion pathways [17].

The alignment of the protein sequence of F3 lipase and other known lipases are shown in Figure 1. The well known consensus sequence AHSMG, and three catalytic residues consisting of serine, aspartic acid and histidine forming the enzyme active site were recognized according to previous reports [3,20]. The results showed that other than the enzyme active site, oxyanion hole and tentative lid sequences were also highly conserved within these strains (Fig. 1). This led us to believe that conformational changes similar to other lipases from *B. pumilus* strains may occur in F3 lipase during enzymatic activity. However, differences

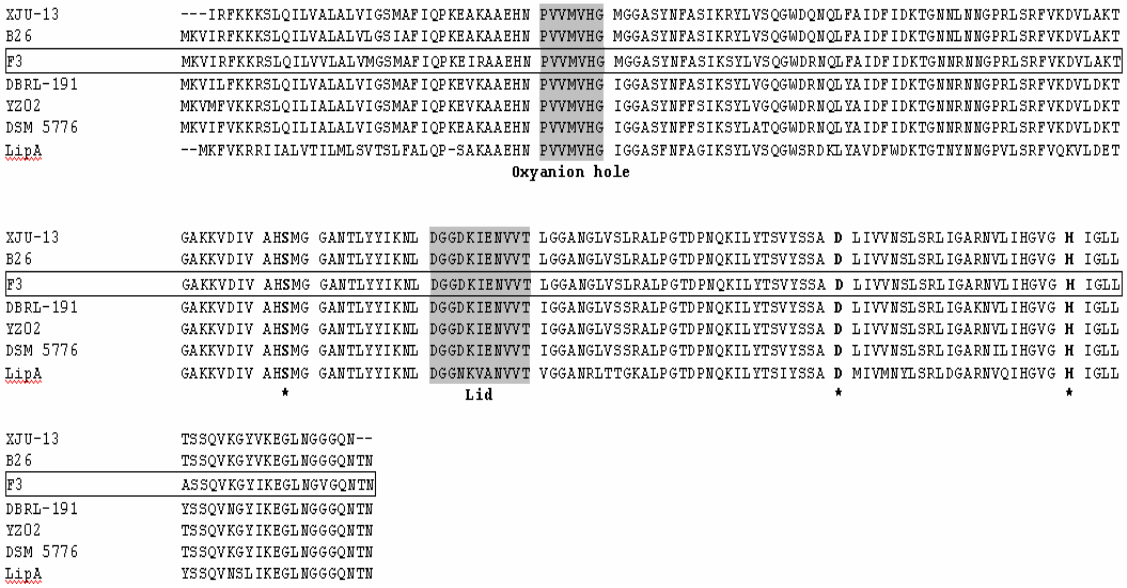


**Figure 1.** Alignment of lipases from *B. pumilus* strains F3, YZ02 (accession number DQ339137), B26 [20], XJU-13 (accession number EF434173), DBRL-191 [30], DSM 5776 [27], and LipA from *B. subtilis* 168 [11] in comparison to LipA. Oxyanion hole and lid residues are highlighted in gray, and catalytic residues are shown by asterisks.

**Table 3.** Lipase activity in different fractions of *E. coli* BL21 harboring pYM124. Production of the recombinant lipase was studied in 100 ml LB culture in 500 ml Flasks with agitation at100 rpm 30°C. The induction was carried out by the addition of 0.2 mM IPTG after 2h of incubation time ($OD_{600}$=0.7)

| Incubation (h) | Fractions | | | | | |
|---|---|---|---|---|---|---|
| | *Periplasmic* | | | *Cytoplasmic* | | |
| | Activity (U/ml) | Protein (mg/ml) | Specific activity (U/mg) | Activity (U/ml) | Protein (mg/ml) | Specific activity (U/mg) |
| 4 | 0.079 | 0.12 | 0.65 | 0.094 | 0.13 | 0.72 |
| 20 | 0.110 | 0.29 | 0.37 | 0.061 | 0.15 | 0.40 |

mainly in signal peptide sequences were observed which may cause different levels of expression in wild type and heterologous host strains. In addition, no cysteines were found in the precursor molecule which demonstrates lack of disulfide bonds in the protein. This could help expression in *E. coli* because the protein need not undergo any conformational changes in the periplasm, while proteins with disulfide bonds need the assistance of periplasmic disulfide bond reductase (Dsb) for their functional folding. On the other hand, the absence of disulfide bond decreases the thermostablity and activity temperature of the enzyme [10,26,28]. Moreover, like *B. pumilus* B26 lipase, no $Ca^{2+}$ binding motif was found near the catalytic histidine site in F3 lipase. Such lipases are more favorable for use in detergents especially in cold washing since they normally contain chelating agents such as EDTA [20]. Other properties of *B .pumilus* lipases are shown in Table 2.

### *Expression of Recombinant Lipase*

To achieve high level expression of lipase F3, pET-26b(+) was used as the expression vector which harbors a strong promoter, T7. The secondary structure of the first 100 nucleotides of the transcripted RNA from pYM124 showed that the ribosomal binding site (RBS) and the start codon were located in a loop harboring 21 nucleic acids. This may enhance the feasibility of concomitant transcription and translation which are coupled in prokaryots [2,32]. Finally, the solubility of the recombinant protein to be expressed in *E. coli* was calculated according to the solubility model used by Koschorreck *et al.*, 2005 [21,41]. The probability of insolubility was 92.12% which shows that the recombinant lipase is hydrophobic enough to form inclusion bodies in *Escherichia coli*.

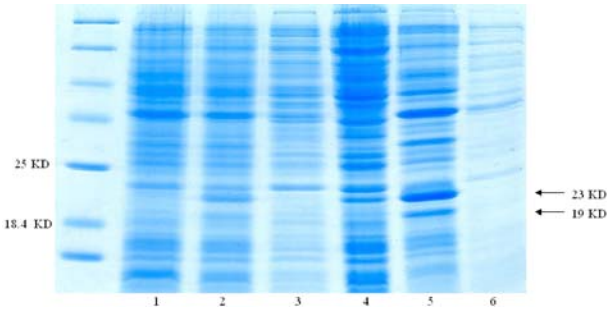


**Figure 2.** The SDS-PAGE analysis of lipase expression in recombinant *E. coli* BL21 containing pYM124. Lane 1, Cell lysate of *E. coli* BL21 containing pET-26b(+) as the negative control; lane 2, total proteins of the cell lysate; lane 3, periplasmic proteins; lane 4, soluble cytoplasmic proteins; lane 5, insoluble cytoplasmic proteins from *E. coli* BL21 harboring pYM124; lane 6, culture medium. As shown, a 23 kDa band is observed in the cell lysate fraction, soluble cytoplasmic proteins and insoluble cytoplasmic proteins. A lighter protein band corresponding to approximately 19 kDa is also observed in the insoluble protein fraction.

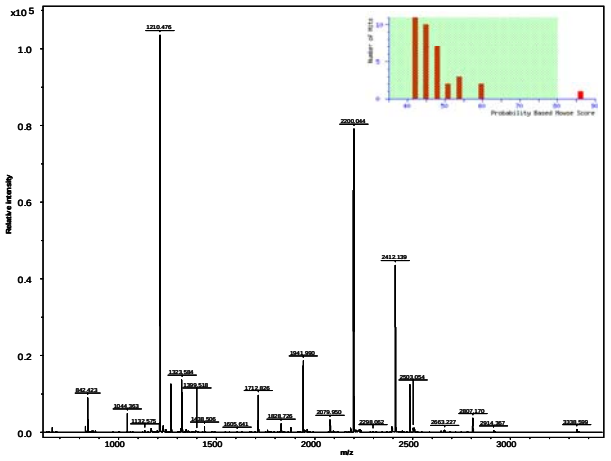


**Figure 3.** Representative MALDI-TOF mass spectrum obtained analysing a 23 kDa band in reflectron mode. The insert shows the MASCOT results, indicating the band to be lipase precursor.

Expression in the general conditions (1 mM IPTG, 200 rpm agitation) resulted in formation of inclusion bodies as revealed by a band of about 23 kDa on SDS-PAGE (data not shown) which confirm the theoretical analysis. The expression level was about 20% of the total cell protein as measured by scanning of the SDS-PAGE by densitometric gel scanner.

In order to avoid formation of inclusion bodies, low concentrations of IPTG, low temperatures and agitation rates were used for expression studies [24]. Cell fractions were then investigated for the presence of 19 (mature lipase) and 23 kDa (precursor protein) bands on SDS-PAGE gels (Fig. 2). No recombinant protein band was observed in the culture medium and periplasmic fractions. The recombinant protein formed inclusion bodies which appeared in the insoluble cytoplasmic protein fraction (Fig. 2). Interestingly, both bands were observed in the insoluble fragment. The expression of *Bacillus* lipases has been suggested to lead to accumulation of inclusion bodies inside the *E. coli* host [24]. However, Rasool *et al.* showed the expression of DBRL-191 lipase in cytoplasm without inclusion body formation [30]. Our results suggest that the formation of inclusion bodies occurred due to high level of expression under the T7 promoter and high hydrophobicity of the protein (92.12%).

Despite the presence of the 19 and 23 kDa bands in the insoluble cytoplasmic protein fraction, low levels of lipase activity was detected in cytoplasm and periplasm (Table 3). Specific activity was equal in cytoplasm and periplasm during different incubation times which suggests that the lipase activity in periplasm could be due to the protein leak. Secretion of *B. pumilus* lipase under its native signal peptide into the periplasmic space of *E. coli* has not been reported so far. Lipase activity decreased after 20 h of incubation in comparison to the 4 h samples (Table 3). This could be due to lipase degradation by cytoplasmic proteases [10]. Presence of the 19 kDa band in the insoluble cytoplasmic protein fraction may indicate partial processing of the recombinant lipase in *E. coli* which could not translacate to periplasm due to its hydrophbic structure.

To ensure the presence of lipase in the excised gel band, in-gel digestion of the 23 kDa band (commonly expressed in the expression experiments) was performed which was followed by Peptide Mass Fingerprinting (PMF) via MALDI-TOF mass spectrometry. The gel band was identified as *B. pumilus* lipase precursor with 215 amino acids, molecular weight of 23135 Da and pI 9.99. The representative PMF spectra of the gel band is shown in Figure 3.

In conclusion, our studies showed high DNA and protein sequence similarity between the F3 lipase and lipases from other *B. pumilus* strains. The F3 lipase was not secreted by its native signal peptide from the cytoplasm in the recombinant host. Further studies are under process to enhance lipase expression in the other expression systems.

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