Periplasmic expression of *Bacillus thermocatenulatus* lipase in *Escherichia coli* in presence of different signal sequences

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Abstract
Efforts to express lipase in the periplasmic space of *Escherichia coli* have so far been unsuccessful and most of the expressed recombinant lipases accumulate in the insoluble cell fraction. To evaluate the role of native and heterologous signal peptides in translocation of the lipase across the inner membrane of *E. coli*, the lipase gene (*btl2*) was cloned downstream of the native *Bacillus* signal peptide and also in fusion with the *pel*B, *ans*B and *ans*B/asp signal peptides. For this purpose, four recombinant expression vectors (pYRK^P.P, pYRK^P.N, pYRK^P. A and pYRK^P.AA) were constructed and expressed in *E. coli.* Osmotic shock analysis showed that recombinant lipase was overexpressed as inclusion bodies in *E. coli*. The lipase inclusion bodies were subsequently solublized, refolded and purified using single column ion-exchange chromatography. To evaluate localization of lipase in the cell, the purified lipases were subjected to capillary isoelectric focusing and tandem mass spectrometry. Results showed that all signal peptides were able to direct the lipase from the cytoplasm into the periplasmic space of *E. coli*, because the periplasmic space of *E. coli* is not suitable for lipase folding, the translocated lipase aggregates in this space as inclusion bodies.
Keywords: *Bacillus thermocatenulatus* Lipase; capillary isoelectric focusing; Inclusion bodies; Periplasmic space; Tandem mass spectrometry

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INTRODUCTION

Lipases (EC 3.1.1.3) are important enzymes, which catalyze the hydrolysis of long chain fatty acid esters of triacylglycerol at the oil-water interface (Schmid and Verger, 1998; Reetz 2002; Gupta and Rathi, 2004). Lipases not only can catalyze hydrolytic reactions but also can catalyze a variety of synthesis reactions including inter-esterification, esterification and trans-esterification (Jaeger and Reetz, 1998; Vakhlu and Kour, 2006). These enzymes play important roles in biotechnology and have been used in different industries such as; food, dairy, textile, surfactant, oil processing, detergent and pharmaceutical (Kirk *et al*., 2002; Jaeger *et al*., 1999). *Escherichia coli* has a periplasmic space which is suitable for recombinant protein expression, due to advantages of periplasmic expression of recombinant proteins, researchers have shown great interest in the expression of lipasewith in this space (Georgiou and Segatori, 2005; Choi and Lee, 2004). In both Gram-positive and Gram-negative bacteria, secreted proteins are initially synthesized with the N-terminal signal peptides, which are removed during translocation across the inner cell membrane (Watson, 1984). Although, efforts are continually being made to express lipase in the periplasmic space of *E. coli*, but all of them have so far been unsuccessful and most of the lipases partition into the insoluble cell fraction (Zelena *et al*., 2009; Hockney,

1994; Rúa, 1997). This study investigated the role of different signal peptides in the translocation of the *Bacillus thermocatenulatus* lipase (BTL2) across the cytoplasmic membrane into the periplasmic space of *E. coli.* Investigation of the cellular localization of lipase inclusion bodies in the *E. coli* was also studied by using osmotic shock, isoelectric focusing and tandem mass spectrometry.

MATERIALS AND METHODS

Bacterial strains, plasmids and cultivation: *Bacillus thermocatenulatus* (DSM 730) was used for genomic DNA preparation. The plasmids pTZ57R/T (Fermentas, Germany) and pET-26b (+) (Novagen, USA) were used for cloning and expression of the lipase gene respectively. *Bacillus thermocatenulatus* was grown as reported previously (Schmidt-Dannert *et al*., 1994) and *E. coli* DH5α and Bl21 strains were grown in LB medium containing ampicillin (100 µg/ml) or kanamycin (30 µg/ml) at 37°C.

Cloning and construction of expression plasmids: Genomic DNA of *B. thermocatenulatus* was isolated according to Sambrook *et al*. (2001). The *btl2* gene along with the native signal peptide was amplified by PCR based on available sequences (GenBank accession no. X95309) using the primer pair N.BTL2.F and BTL2.R (Table 1). The PCR product (1279 bp) was cloned into pTZ57R/T resulting in the pYRK^{T}-N plasmid.

The recombinant pYRK^{P}.P plasmid was constructed by cloning the *btl2* gene downstream of the *pel*B signal peptide. The *btl2* gene was amplified by using the primer pair, BTL2.F and BTL2.R (Table 1). The PCR product (1198 bp) was cloned into pTZ57R/T leading to the pYRKT.P plasmid.

The *ans*B signal peptide was added to the *btl2* gene by using a two-steps PCR procedure. The first PCR was carried out using the primer pair *ans*B. BTL2.F1 and BTL2.R (Table 1). Then the PCR product (1222 bp) was extracted and used for the second round of PCR using the primer pair, ansB.F2 and BTL2.R (Table 1). Subsequently, the PCR product (1261 bp) was cloned into pTZ57R/T leading to the formation of the pYRK^{T}.A plasmid.

The recombinant pYRK^{P}.AA plasmid was also constructed by using two-steps PCR. In the first PCR, the DNA fragment encoding the first 10 amino acid residues of asparaginase was added to the *btl2* gene using the primer pair, ans.asp.BTL2.F1 and BTL2.R (Table 1). In the second PCR, the *ans*B signal peptide was added to the first PCR product by using the primer pair of AnsB.F2 and BTL2.R (Table 1). The resulting PCR product (1291bp) was then cloned into the pTZ57R/T leading to formation of the pYRK^{T}.AA plasmid. Subsequently, the pYRK^{T}.P plasmid were digested with *Mlu* NI and *Sac* I, and the pYRK^{T}.N, pYRK^{T}.A and pYRK^{T}.AA plasmids were digested with *Nde* I and *Sac* I, and the resulting fragments were then ligated into the corresponding restriction sites of the pET-26b(+) to produce pYRK^{p}.P, pYRK^{p}.N, pYRK^{p}.A pYRK^{P}.AA respectively. Finally, the four

Table 1. The sequences of primers were used in this study. The enzyme sites are bolded and underlined.

Primer name	Nucleotide sequence	RE[a]
BTL2.F	5'-GATGGCCATGGCGGCATCCCCACGCGCC-3'	*Mlu NI*
BTL2.R	5'-TTGAGCTCATCATCCCTTCATTAAGGCCGC-3'	*Sac I*
N.BTL2.F	5'-ATCATATGATGAAAGGCTGCCGGGTGATGG-3'	*Nde I*
ans.BTL2.F1	5'-GGTGCAGCATTGGCAGCGGCATCCCCACGCGC-3'	
ans.F2	5'-TACATATGGAGTTTTTCAAAAAGACGGCACTTGCC GCACTGGTTATGGGTTTTAGTGGTGCAGCATTGGCA-3'	*Nde I*
ans.Asp.BTL2.1	5'-AGTGGTGCAGCATTGGCATTACCCAATATCACCATT TTAGCAACCGGCGCGGCATCCCCACGCGC-3'	

[a]Restriction enzyme (RE).

recombinant expression plasmids were introduced into *E. coli* Bl21 (DE3) for expression of the recombinant lipase.

Expression and purification of recombinant lipases: Expression and purification of recombinant lipases were performed as previously described (Karkhane *et al*., 2009). Briefly, 2.5 ml of an over night culture of *E. coli* harboring different recombinant plasmids were inoculated into 250 ml of LB medium supplemented with kanamycin (30 µg/ml) and incubated at 37°C with shaking at 220 rpm, until an optical density (OD_{600}nm) of 0.6 was reached. Subsequently, IPTG (0.2 mM) was added to induce protein expression at 37°C for 2.5 h (220 rpm) with additional shaking (150 rpm) at 30°C for 16 h and *E. coli* cells were then harvested by centrifugation at 2500 rpm for 10 min at 4°C (J-6 centrifuge, rotor # 4.2, Beckman, USA). The cells were re-suspended in lyses buffer (20 mM Tris. HCl (pH 8.5), 0.5% (v/v) Triton X-100, 10 mM EDTA, 100 mM NaCl, 1 mM PMSF, and 1 mM DTT), supplemented with 10 µg/ml of lysozyme and 100 µg/ml of DNase and then stirred for 30 min at room temperature. The lysate was sonicated three times (3×1 min) at maximum power and centrifuged (10,000 g, 4°C, 10 min). The supernatant was separated and the pellet (inclusion bodies) was washed three times with washing buffer (20 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.5).

The inclusion bodies were resuspended in solubilization buffer (20 mM Tris-HCl (pH 8.5), 8 M urea, 10 mM DTT) at a final concentration of 5 mg/ml, stirred for 1 h at room temperature and then centrifuged (10,000 g, 4°C, 10 min). The supernatant was separated, and diluted drop wise to a final protein concentration of 0.1 mg/ml with refolding buffer (400 mM L-arginine monohydrochloride, 100 mM Tris-HCl, pH 8.5, 100 mM NaCl, 100 mM glycine, 10 mM DTT, 5% (v/v) glycerol). The protein solution was dialyzed against dialysis buffer (20 mM Tris-HCl, pH 8.5) with three exchanges of buffer. Ultimately, insoluble materials were removed by centrifugation at 10,000 g for 10 min at 4°C.

The wild type and mutant lipases were purified using one-step ion-exchange chromatography (Rastgar Jazii *et al*., 2007). The crude lipase solution was dialyzed (20 mM Tris.HCl, pH 6.8) at 4°C with three exchanges of buffer. The dialyzed solution was passed through a DE-52 cellulose column (Whatman, Maidstone, England) pre-equilibrated with dialysis buffer, and the unbounded proteins were immediately collected as purified lipase. The purified lipases were dialyzed against dialysis buffer (10 mM Tris-HCl, pH 8.5) at 4°C with three exchanges of buffer and was cheeked by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

Osmotic shock: Osmotic shock was performed according to the method of Neu and Heppel (1965), but with some modifications. Briefly, bacterial culture was centrifuged at 4000 rpm for 10 min at 4°C, the pellet was suspended in 15 ml of TES buffer (0.2 M Tris-HCl pH 8.0, 0.5mM EDTA, 0.5M sucrose) per liter of the original culture and was shacked for 20 min on ice. Subsequently, 22.5 ml cold water containing 2 mM $MgCl_2$ and 1mM PMSF was added, incubated on ice for 30 min, centrifuged at 20,000 rpm (Sigma, UK) for 20 min, and the periplasmic fraction (supernatant) was then collected.

Lipase assay: Lipase activity was determined by the pH-stat assay (842 Titrando, Metrohm Ltd, Herisau, Switzerland) at 55°C (pH 8.5) in the presence of different triacylglycerol (C_4 to C_{18}) substrates, as described (Karkhane *et al*., 2009). Briefly, 0.5 µg (10 µl) of the purified lipase was added to the lipase substrate and liberated fatty acids were titrated automatically with 0.05 M NaOH for 5 min. Lipase activity was calculated by the amount of NaOH needed to maintain the pH at 8.5. One unit was defined as the amount of enzyme that released 1.0 µmoles of fatty acid per min (Quyen *et al*., 2003).

Capillary isoelectric focusing: Capillary isoelectric focusing was performed according to the method of O'Farrell (1975), as described previously (Rastgar Jazii *et al*., 2007).

Mass spectrometry (MS): Purified recombinant Lipase was subjected to SDS-PAGE and visualized by Coomassie staining. The gel band was excised and used for mass spectrometry analysis. Mass spectrometry (MS+MS/MS) was performed using a 4800 MALDI TOF/TOF analyzer (Applied Biosystems, USA) in the Core Facility for Proteome Analysis at the University of Antwerpen (Germany).

RESULTS

Construction of the expression cassettes and lipase expression: To study the role of signal peptides during

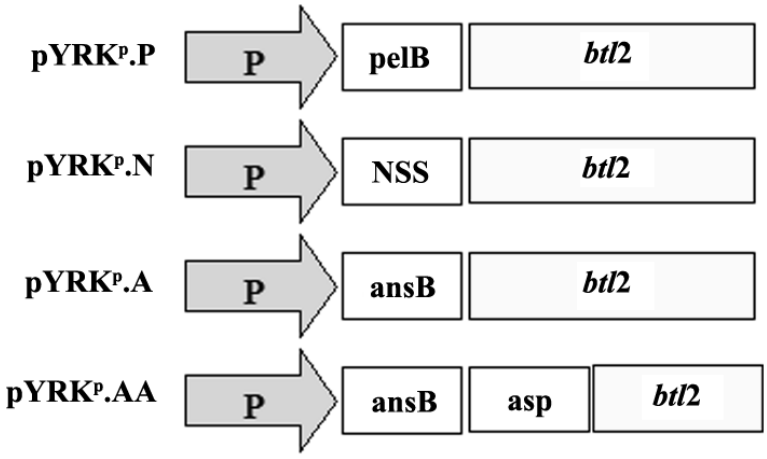

Figure 1. Schematic representation of the recombinant expression cassettes (pYRKP.P, pYRK^P.N, pYRK^P. A and pYRK^P.AA vectors) derived from the pET26 b(+) vector. P: promoter; *pel*B: *pel*B signal peptide; NSS: native signal peptide; *ans*B: *ans*B signal peptide; asp: first 10 amino acid residues of the asparaginase enzyme; *btl2*: mature lipase gene.

periplasmic expression of the *B. thermocatenulatus* lipase (BTL2) in *E. coli*, a set of expression cassettes with different signal peptides was constructed (Fig. 1). To express the *btl*2 gene along with the *pel*B signal peptide, the lipase gene, was amplified by PCR and cloned into the pPET26b(+) vector downstream of the *pel*B signal peptide under control of the T7 promoter leading to formation of the recombinant pYRK^P.P vector. Subsequently, the recombinant pYRK^P.P vector was introduced into *E. coli*, and the expression of the recombinant lipase was induced by adding IPTG and the cells were the subjected to osmotic shock. SDS-PAGE analysis of the whole cell lysate and osmotic shock fractions (Fig. 2A) showed that the lipase protein was over-expressed (Fig. 2A, line 2), but most of the expressed lipase remained in the insoluble cytoplasmic cell fraction (Fig. 2A, line 4) and a very faint band associated with the expressed recombinant lipase was seen in the soluble cell fraction (Fig. 2A, line 3). Protein misfolding or unfolding and over-expression of the recombinant genes are common events which can lead to the formation of large insoluble aggregates (inclusion bodies) (Wang *et al*., 2008; Wickner *et al*., 1999; Arié *et al*., 2006). Also, inclusion body formation may have resulted from intermolecular hydrophobic interactions between the hydrophobic regions of the participating proteins (Arié *et al*., 2006; Fink, 1998).

It has been previously reported that a non-*E. coli* signal peptide may lead to the expression of low levels of the recombinant protein (Yazdani and Mukherjee, 1998). So, in order to decrease the expression levels of lipase, the lipase gene with its native *Bacillus* signal peptide (pYRK^P.N vector) was cloned and expressed in *E. coli* (Fig. 2B). In this case, although, the *Bacillus* signal peptide led to a decrease in lipase expression levels (Fig. 2B, line 2), most of the expressed lipase was seen in the insoluble cell fraction (Fig. 2B, line 4)

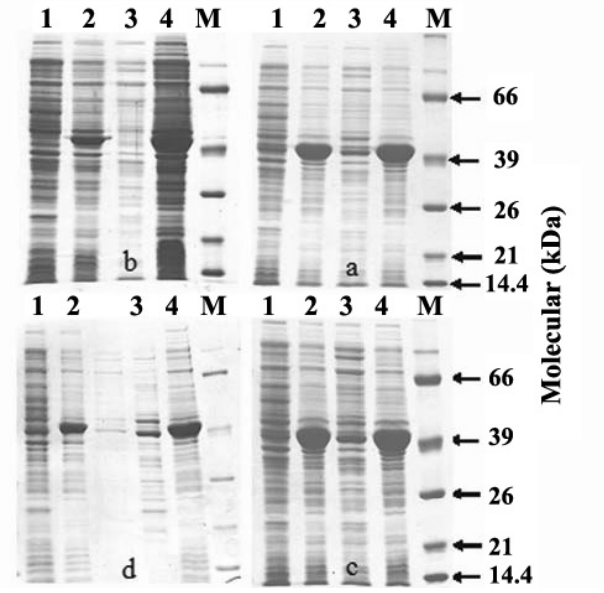

Figure 2. SDS-PAGE analysis of the expressed recombinant lipase in *E. coli* harboring different expression vectors ; (a) pYRK^P.P , (b) pYRK^P.N, (c) pYRK^P.A and (d) pYRK^P.AA vector. Lanes 1 and 2, crude cell extract of recombinant clones before and after induction respectively; Lanes 3 and 4, soluble and insoluble fraction obtained from the osmotic shock prcedure respectively; Lane M, molecular weight marker (SM 0431, Fermentas).

and very little of the lipase was presented in a soluble form (Fig. 2B, line 3).

To further study the role of the signal peptide in transportation of the lipase across the cytoplasmic membrane into the periplasmic space, the lipase gene (*btl2*) along with the *ans*B signal peptide (pYRK^P.A vector), an *E. coli* specific signal peptide was cloned and expressed in *E. coli*. Subsequently, secretion of the lipase into the periplasmic space was studied with osmotic shock method. SDS-PAGE analysis of the osmotic shock fractions showed (Fig. 2C) similar results obtained when using the pelB signal peptide (Fig. 2C, line 4); only some of the lipase was able to export into the periplasmic space (Fig. 2C, line 3). In the next step, it was concluded that in addition to the compatibility of the signal peptide with the *E. coli* secretion system, but also the first amino acid residues of the protein of interest could also be important for transport of the protein across the cytoplasmic membrane into the periplasmic space. In this case, the DNA encoding the *ans*B signal peptide along with the first 10 amino acid residues of L-asparaginase II was added to the 5' end of the mature lipase gene (pYRK^P.AA vector), and resulting fusion gene was the cloned and expressed in *E. coli* (Fig. 2D). SDS/PAGE analysis of fractions obtained by osmotic shock preparations was similar to the results obtained when previous constructs were used, in that most of the expressed lipase was in the insoluble fraction (Fig. 2D, line 3) and only a very little amount of the lipase was present in the soluble cell fraction (Fig. 2D, line 4). The results obtained from all constructs showed that the lipase protein was expressed as inclusion bodies within the cell.

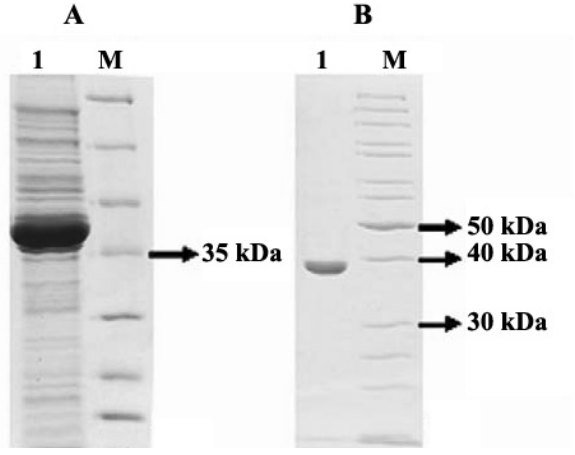

Figure 3. SDS-PAGE analysis of *E. coli* harboring recombinant pYRK^P.P plasmid. Lane 1A; lipase inclusion bodies, lane 1B; purified recombinant lipase and lane M; molecular weight marker.

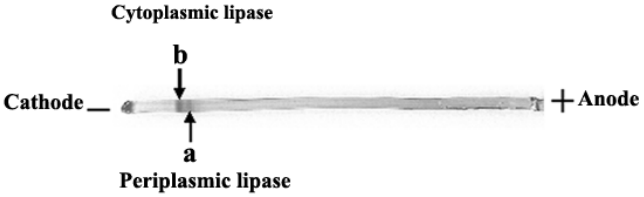

Figure 4. Tube capillary isoelectric focusing analysis of the purified recombinant lipase. A: cytoplasmic recombinant lipase with the signal peptide (pI of 6.50). B: periplasmic recombinant lipase without the signal peptide (pI of 6.37).

Mass spectrometry and isoelectric focusing (IEF): To evaluate cellular localization of the recombinant lipase inclusion bodies in *E. coli*, the purified recombinant lipase was analyzed with tandem mass spectrometry (MS+MS/MS) and IEF techniques. The recombinant lipase inclusion bodies from *E. coli* harboring the pYRK^P.P plasmid were isolated (Fig. 3A), solubilized, refolded and purified using one step ion-exchange chromatography (Fig. 3B) (Karkhane *et al*., 2009). The

Table 2. The amino acid sequence of purified lipase by mass spectrometry (MS+MS/MS).

a	b	c	d
MW	Start Sequence	End Seqsequence	Sequences
1823.8306	33	49	ANDAPIVLLHGFTGWGR
1823.8306	5	21	ANDAPIVLLHGFTGWGR
1679.7052	48	62	TYTLAVGPLSSNWDR
1679.7052	76	90	TYTLAVGPLSSNWDR
1579.5978	64	76	GDIEQWLNDNGYR
1579.5978	35	47	GDIEQWLNDNGYR
1286.6294	93	103	TYPGLLPELKR
1286.6294	122	132	TYPGLLPELKR
1474.6711	136	149	VHIIAHSQGGQTAR
1474.6711	107	120	VHIIAHSQGGQTAR
1604.6439	149	162	MLVSLLENGSQEER
1604.6439	121	134	MLVSLLENGSQEER
1043.4757	237	244	LDQWGLRR
1043.4757	208	215	LDQWGLRR
1220.4786	260	270	SPVWTSTDTAR
1220.4786	231	241	SPVWTSTDTAR
2404.0635	280	299	LNQWVQASPNTYYLSFS
2404.0635	251	270	LNQWVQASPNTYYLSFS
1667.6466	243	255	RQPGESFDHYFER
1667.6466	214	226	RQPGESFDHYFER
1097.53	408	417	LAEQLASLRP
1097.53	379	388	LAEQLASLRP

purity of purified recombinant lipase was checked by isoelectric focusing (Fig. 4) and then subjected to mass spectrometry and IEF analysis.

An assessment of the amino acid sequences from recombinant lipase obtained by tandem mass spectrometry (MS+MS/MS) revealed that amino acid sequences, but with differences in the starting points of the amino acid sequences (Table 2, columns b, c and d). The difference between the starting points of the amino acid sequences belonging to the identical peptides is 28 amino acids, which is equal to the signal peptide plus 6 amino acids from the N-terminal end of the BTL2 lipase.

In the capillary IEF gel, two bands close to each other were observed. This result indicate that the purified recombinant lipase inclusion bodies contain two lipases with smoll differences in mass and pI values. Theoretical valuessfor the isoelectric point (pI) and molecular weights (Mw) of the recombinant cytoplasmic lipase (with the signal peptide) and the recombinant periplasmic lipase (without signal peptide) were estimated using Compute pI/Mw tool [http://www.expasy.org/tools/pi_tool.html] to be 6.50 / 45408 Da and 6.37 / 43197.38 D respectively.

Also, alignment of the amino acid peptides of all fragments obtained with combined mass spectrometry using the NCBI database, strongly confirmed that the produced lipase belongs to *Bacillus thermocatenulatus* (Table 3).

Lipase activity: The activity of the purified lipase was determined in a pH-stat (55°C at pH 8.5) in the presence of different triacylglyceride substrates (C4-C18). Figure 5 shows that purified recombinant lipases are active in the presence of all substrate with maximum activity towards the tributyrin substrate (C4) which previously reported (Quyen *et al*. 2003). Also, the presence of the unprocessed signal peptides may no interfere with enzyme activity.

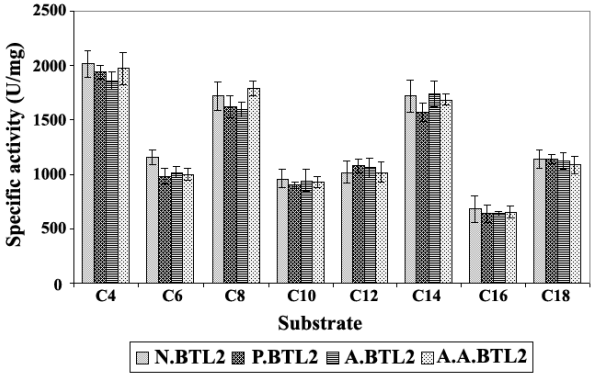

Figure 5. Activity and substrate specificity of the recombinant lipases. Activity was measured at 55°C and pH 8.5.

DISCUSSION

Most of the Gram-negative bacteria require a lipase-specific foldase (Lif) in order to properly fold lipases in the periplasmic space (Schmidt-Dannert *et al*., 1996). In the lipase producing Gram negative bacteria, lipases are transported into the periplasmic space via the Sec pathway and often require the assistance of specific molecular chaperones such as the lipase-specific foldases, to properly fold in the periplasmic space (Schmidt-Dannert *et al*., 1996; El Khattabi *et al*., 1999; Missiakas and Raina, 1997). After cleavage of the N-terminal signal peptide and folding in the periplasmic

Table 3. The alignment of the amino acid sequences of purified lipase derived from pYRK^{P}.P plasmid subjected to combined mass spectrometry (MS+MS/MS) using NCBI database.

Spot Label	Rank	Protein Name	Accession No.
pYRKP.P	1	triacylglycerol lipase [Geobacillus thermocatenulatus]	gi|1321706
pYRKP.P	2	thermostable organic solvent tolerant lipase [Bacillus sp. 42]	gi|54609997
pYRKP.P	3	chain A, structure of the Bacillus Stearothermophilus L1 lipase	gi|23200288
pYRKP.P	4	lipase [Geobacillus stearothermophilus]	gi|3015530
pYRKP.P	5	chain A, crystal structure of the Bacillus Stearothermophi	gi|24987388
pYRKP.P	6	thermostable lipase [Bacillus sp. L2]	gi|57232539
pYRKP.P	7	chain A, crystal structure of T1 lipase F16l mutant	gi|159795726
pYRKP.P	8	lipase [Geobacillus thermoleovorans]	gi|4835874
pYRKP.P	9	lipase [Geobacillus sp. SF1]	gi|67906830
pYRKP.P	10	triacylglycerol lipase [Bacillus sp. TP10A.1]	gi|7532786

space, the lipase is then secreted into the extracellular medium via the secreton transporter complex.Due to advantages associated with the expression of recombinant proteins in the periplasmic space of *E. coli*, there is currently much interest to express proteins within this space. However, all efforts carried out so far have been unsuccessful and most of the lipases are fractionated into the insoluble cell fraction. To evaluate the role of signal peptides in transportation of the lipase into the periplasmic space, four recombinant vectors based on different signal peptides were constructed. Osmotic shock analysis showed that the recombinant lipase was expressed as insoluble inclusion bodies in *E. coli*. Müller *et al*. (1989) have previously reported that the incomplete secretion of recombinant proteins into the periplasmic space of *E. coli* may be due to the incompatibility of the signal peptide with the *E. coli* secretion system.

Although, capillary IEF and MS data showed that all signal peptides could pass through the lipase from the cytoplasm into the periplasmic space, but, since the periplasmic space of *E. coli* is unsuitable for lipase folding, the translocated lipase that aggregates in this space would be seen in the insoluble fraction during the cell fractionation experiments. Results of the mass spectrometry technique confirmed the results obtained by isoelectric focusing and showed that two kinds of lipase inclusion bodies were produced that were localized not only in the cytoplasmic space but also in the periplasmic space of *E. coli*. Part of the expressed lipases remained in the cytoplasmic space as inclusion bodies and yet contained the signal peptide, whereas, the other part of the expressed protein was secreted into the periplasmic space following cleavage of the N-terminal signal peptide.

CONCLUSIONS

In cunclusion, the combined mass spectrometry approach showed that all of the signal peptides were able to direct the lipase protein through the cytoplasmic membrane and into the periplasmic space of *E. coli*. Since *E. coli* dose not produce lipase, it does not have the lipase-specific foldases in the periplasm membrane which is necessary for lipase folding. As a result, *E. coli* may be unable to fully fold the lipase, resulting in the aggregation of lipase in the periplasmic space as inclusion bodies (Rúa *et al*., 1997). In addition, the presence of hydrophobic regions in lipase dramatically increases the tendency of lipase to form insoluble aggregates in both the cytoplasm and periplasmic space (Lang and Dijkstra, 1998; Lang *et al*., 1998).

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