IJFANS INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES
ISSN PRINT 2319 1775 Online 2320 7876
 Journal UGC CARE Listed (Group-I) Volume 11, Issue 01 2022

Molecular Characterisation of an Uncommon Enantioselective Lipase TALipA from *Trichosporon asahii* MSR54, Highlighting the Discovery of an AXSXG Signature Sequence

***[1]Abhishek Chauhan, and [2]Dr. Ruby Gupta**

***[1]Research Scholar, Department of Microbiology**

Malwanchal University Indore, Madhya Pradesh

ABSTRACT: A lipase-encoding gene, designated as TALipA, was effectively produced in *Pichia pastoris* X-33 after being cloned from *Trichosporon asahii* MSR54. Using affinity chromatography, the enzyme was purified with a purification fold of 1.8, and SDS-PAGE analysis confirmed it as a monomeric protein of 27 kDa. Close resemblance to bacterial and actinobacterial lipases was revealed by comparative sequence analysis, while having distinctive characteristics like a conserved AHSMG pentapeptide, where alanine replaces glycine—a rare feature among yeast lipases—and an uncommon oxyanion hole "GL." The enzyme was most active at 60 °C and pH 8.0, and it remained stable with a half-life of 68 minutes at 70 °C. TALipA displayed substrate specificity toward long-chain *p*-nitrophenyl esters, especially p-np palmitate, which was verified during the hydrolysis of triacylglycerides. Hydrolysis of triolein revealed regioselective behavior, while esterification of phenylethanol demonstrated solvent-dependent enantioselectivity—preferring the R-enantiomer in isopropanol and hexane and the S-enantiomer in 1,4-dioxane. The lipase was further identified as a magnesium-activated metalloenzyme, with stability in the majority of polar and non-polar solvents but activity inhibited by 10 mM EDTA. These findings suggest that TALipA represents a novel yeast lipase with significant potential as a biocatalyst for industrial applications.

Keywords: TALipA, *Trichosporon asahii* MSR54, *Pichia pastoris* X-33, metalloenzyme, enantioselective lipase

Introduction

Hydrolytic enzymes known as lipases (EC 3.1.1.3) are crucial to biotechnology due to their chemo-, regio-, and enantioselective characteristics. Owing to these unique catalytic features, they are highly valued in industries such as oleochemistry, pharmaceuticals, agrochemicals, and nutraceuticals [1]. Numerous microbial lipases, such as those produced by yeasts and bacteria, are already commercially available for industrial biotransformations, with yeast-derived lipases being among the most extensively utilized [2].

So far, Only a few yeast species, such as *Candida sp., Cryptococcus sp., Geotrichum candidum, Galactomyces geotrichum, Yarrowia lipolytica*, and *Trichosporon fermentans*, have been used for the large-scale production of lipase [2]. Recent investigations on *Trichosporon asahii* MSR54 reported the release of two extracellular lipases (Lip 54 and Lip 27 kDa), which displayed characteristics like enantioselectivity and regioselectivity that are relevant to industry [3, 4].

However, since *T. asahii* is known to be pathogenic, direct large-scale exploitation for lipase production is not feasible. Therefore, an alternative approach is to isolate and clone the genes encoding these lipases into a non-pathogenic heterologous host to enable safe and effective overproduction for use in industry.

The 27 kDa lipase gene, TALipA, was isolated, its sequence was analysed, and it was expressed heterologously in *Pichia pastoris* X-33. Additional characterization of the recombinant enzyme was done to evaluate its catalytic and functional properties.

Materials and Methods

Materials

The laboratory culture collection provided the *Escherichia coli* DH5α and the yeast strain *Trichosporon asahii* MSR54. SRL (India) supplied all of the chemicals and reagents needed to prepare the buffer and solution. Invitrogen provided the host strain Pichia pastoris X-33, the expression vector pPICZαA, and the antibiotic zeocin. Qiagen provided the gel extraction and plasmid purification kits. Sigma Aldrich (USA) supplied the lipase substrates, which included p-nitrophenyl (p-NP) esters and triacylglycerides. Hi-Media (India) provided media ingredients like Luria Bertani broth, tryptone, yeast extract, yeast nitrogen base, and methanol.

Reverse Genomics for Gene Isolation

Following the purification of the 27 kDa lipase (TALipA) from T. asahii by Q-sepharose chromatography, N-terminal sequencing, and LC/MS-MS analysis [4], peptide sequences discovered through BLAST searches were found to be similar to an extracellular lipase of T. asahii CBS 89004, and lipase-specific primers were designed from the terminal regions of the gene based on the sequence that was available. Using *T. asahii* MSR54 genomic DNA as a template, the TALipA gene was amplified with primers containing EcoRI and NotI restriction sites (forward primer: 5′-GAATTCATGCGTCTCTCCCTAGTGACCGCC-3′; reverse primer: 5′-GCGGCCGCGATCGCTAAGAAGAATGG-3′). After being cloned into the PCR4 vector, the PCR product was sequenced. Following colony PCR and sequencing confirmation of positive clones, EcoRI and NotI digestion was followed by subcloning into pPICZαA. After transforming the recombinant plasmid into *E. coli* DH5α, transformants were chosen using LB agar supplemented with 25 µg/ml of zeocin. Qiagen maxi-prep kits were used to isolate plasmids.

Transformation in Pichia pastoris

Using the lithium acetate method, the recombinant construct pPICZαA-TALipA was linearised with PmeI and changed into P. pastoris X-33 [5]. Following three days of incubation, colonies were moved to YPD plates with varying zeocin concentrations (50–200 µg/ml) (1% yeast extract, 2% peptone, 2% dextrose). To find colonies that produced lipase, clones that grew at 200 µg/ml were further screened on tributyrin agar (1.5% tributyrin, 2% agar).

Expression in the Pichia pastoris

In YPD medium, recombinant P. pastoris containing the TALipA gene was cultivated overnight until its OD700 reached 11.0. After that, the cells were placed in BMGY medium, which contained 2% yeast extract, 3% peptone, 2% glycerol, 150 mM potassium phosphate buffer, pH 8.0, and 1.35% yeast nitrogen base. They were then allowed to grow until the OD700 reached 7.0. OD600 = 3.0 was achieved by harvesting the cells, resuspending them in BMMY medium (2% yeast extract, 3% peptone, 150 mM potassium phosphate buffer pH 8.0, and 1.35% yeast nitrogen base). The incubation process was run at 250 rpm and 35 °C. To promote protein expression, methanol (0.6%) was added after three hours and then every twenty-four hours.

Assay for Lipase and Protein Estimation

Olive oil was used as the substrate in a titrimetric assay to confirm the lipase activity, which was measured using p-NP palmitate [4, 6]. Under ideal assay conditions, one unit of lipase activity was defined as the amount of enzyme that released one µmol of p-nitrophenol or fatty acid per millilitre per minute. Using bovine serum albumin as the standard, the Bradford method was used to calculate the total protein content.

Enzyme Purification and Biochemical Characterization

Ni2+-NTA affinity chromatography was used to purify the recombinant enzyme. SDS-PAGE was used to analyse purity. Western blotting and zymography were used to further validate the enzyme activity, as described by Kumari and Gupta (2013) [5].

Biochemical Studies

Using established protocols, the effects of pH and temperature on enzyme stability and activity were ascertained [7]. In order to assess thermostatability, the enzyme was incubated at various temperatures for 20–120 minutes. Kd, t1/2, and D-values were then calculated as previously reported [8].

Effects of Solvents, Metal Ions, and Chelators

Various solvents (50 v/v) such as isopropanol, tetrahydrofuran, DMSO, hexane, butanol, ethyl acetate, acetone, petroleum ether, and ethanol were used to test the enzyme activity. The effects of metal ions (Ca^{2+}, Mg^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, Mn^{2+}; 1–20 mM), EDTA, and EGTA were also studied. After 1 h of incubation, residual enzyme activity was measured relative to a control sample without additives. Fluorescence spectroscopy was used to study the reversal of EDTA inhibition by Mg^{2+}.

Substrate Specificity and Selectivity

p-NP esters were used to analyse substrate specificity and an equimolar triacylglyceride mixture (tricaprylin, tricaprin, trilaurin, trimyristin, and tripalmitin) in isopropanol. Reactions were conducted at 50 °C for different time intervals (10–30 min) and analyzed by gas chromatography.

Regio- and Enantioselectivity

By hydrolysing the triolein emulsion and then using gas chromatography, regioselectivity was assessed [7]. Racemic phenylethanol was esterified with myristic acid in various solvents to investigate enantioselectivity. Reactions were incubated at 50 °C overnight, and products were separated and analyzed by HPLC using a chiral column [3]. Peak area was used to calculate enantiomeric excess (ee).

Analysis of Statistics

Every experiment was carried out with duplicate samples in triplicate. The findings were presented as mean ± SD. Sigma software was used to conduct the statistical analysis.

Results and Discussion

Lipase gene cloning

Using genomic DNA from *T. asahii* MSR54, a fragment of approximately 816 bp was successfully amplified with the designed primers. After being cloned into a PCR 4.0 vector, the PCR product was sequenced. The sequence shared 98% homology with a lipase gene (EKD04429) from *T. asahii* CBS 8907, according to BLAST analysis. The sequence was deposited in the NCBI database (Gene ID: KC732450). Bioinformatic analysis revealed the absence of introns. The amplified gene was later subcloned into the pPICZαA vector for heterologous expression.

Sequence and Phylogenetic Analysis

The EXPASY tool was used to convert the nucleotide sequence into its amino acid sequence. Signal peptide analysis predicted a 17-residue sequence at the N-terminus, yielding a mature protein of 255 amino acids (26.8 kDa). BLAST analysis revealed similarity with bacterial lipases, particularly *Streptomyces coelicolor* (55% homology) and *Bacillus subtilis* lipase B (44% homology). Comparisons with the NCBI NR database also revealed significant similarity with lipases from *Amycolatopsis azurea*, *Streptomyces tsukubaensis*, *Rhodococcus*, and *Bacillus* species. Interestingly, structural homology was highest with *Bacillus subtilis* lipase (PDB ID: 1I6WA). At positions 47–48 and 114–118, multiple sequence alignment identified a conserved AHSMG pentapeptide and a distinct oxyanion hole (GL), respectively. Unlike yeast lipases, this conserved AXSXG motif resembles bacterial lipases, indicating that TALipA may represent a novel yeast lipase with bacterial-like features. Phylogenetic analysis confirmed this observation by clustering TALipA closer to bacterial lipases than to other yeast lipases.

Expression and Purification

The *TALipA* coding sequence was inserted into the pPICZαA vector and transformed into *P. pastoris* X-33. Recombinant colonies were selected on zeocin, and successful expression was achieved in BMMY medium supplemented with methanol. The enzyme was secreted extracellularly and purified by affinity chromatography with a 1.7-fold purification and 49% yield. SDS-PAGE, Western blotting, and zymogram analysis confirmed the presence of a 27 kDa monomeric protein.

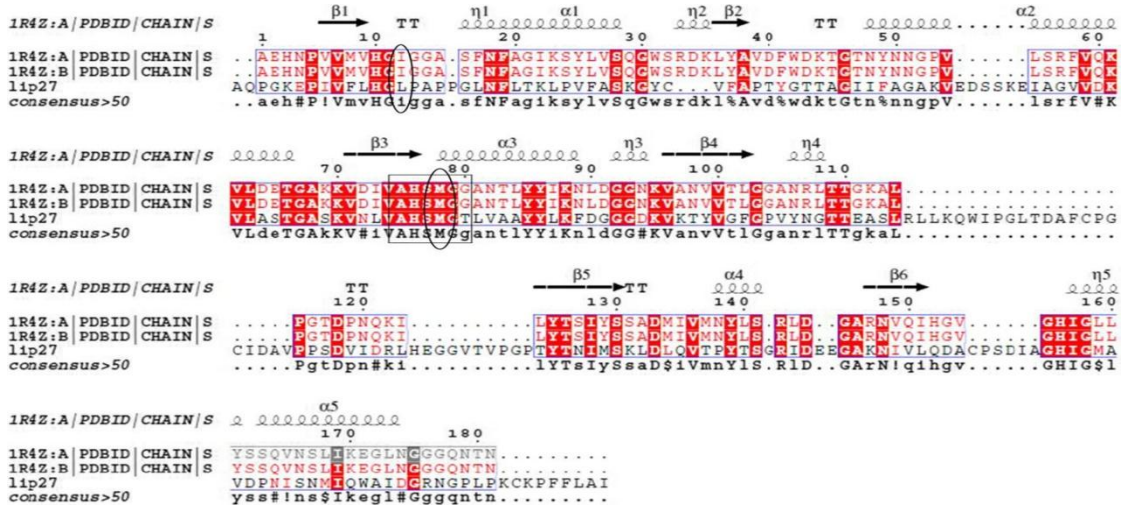


Fig. 1 *Trichosporon asahii* MSR54's TALipA multiple sequence alignment with Bacillus subtilis lipases (LipA and LipB). Rectangular boxes highlight conserved signature residues, while oval shapes denote oxyanion hole residues, and the secondary structures (α-helices and β-strands) are shown above the alignment

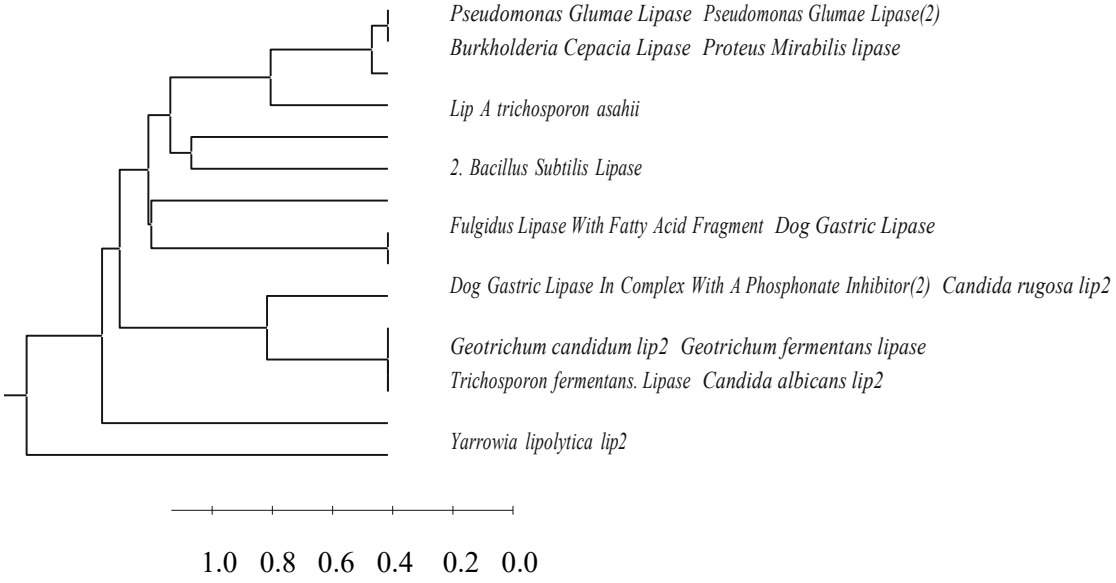


Fig. 2 Phylogenetic tree of TALipA compared with representative lipases. The lipases included in the analysis with their respective Gene/PDB IDs are: *Bacillus subtilis* lipase (gi|14488511|pdb|1I6W|A, chain A), *Fulgidus* lipase (gi|240104284|pdb|2ZYS|A, chain A), dog gastric lipase (gi|20150725|pdb|1K8Q|A, chain A), dog gastric lipase in complex with a phosphonate inhibitor (gi|20150725|pdb|1K8Q|A, chain A), *Proteus mirabilis* lipase (gi|448262557|pdb|4GW3|A, chain A), *Pseudomonas glumae* lipase (gi|5107664|pdb|1QGE|D), *Pseudomonas glumae* lipase 2 (gi|5107664|pdb|1QGE|B, chain B), *Burkholderia cepacia* lipase (gi|67464316|pdb|1YS1|X, chain X), *Candida rugosa* Lip2 (P32946), *Candida albicans* Lip2 (Q9P8V9), *Geotrichum candidum* lipase (P17573), *Geotrichum fermentans* lipase (P79066), and *Yarrowia lipolytica* Lip2 (Q9P8F7)

The recombinant strain of *P. pastoris* X-33 harboring the pPICZαA-TALipA construct secreted lipase into the culture medium (BMMY) when incubated at 30 °C with shaking at 250 rpm (Fig. 3). Affinity chromatography was then used to purify the enzyme, yielding a 49% recovery yield and a 1.7-fold purification.

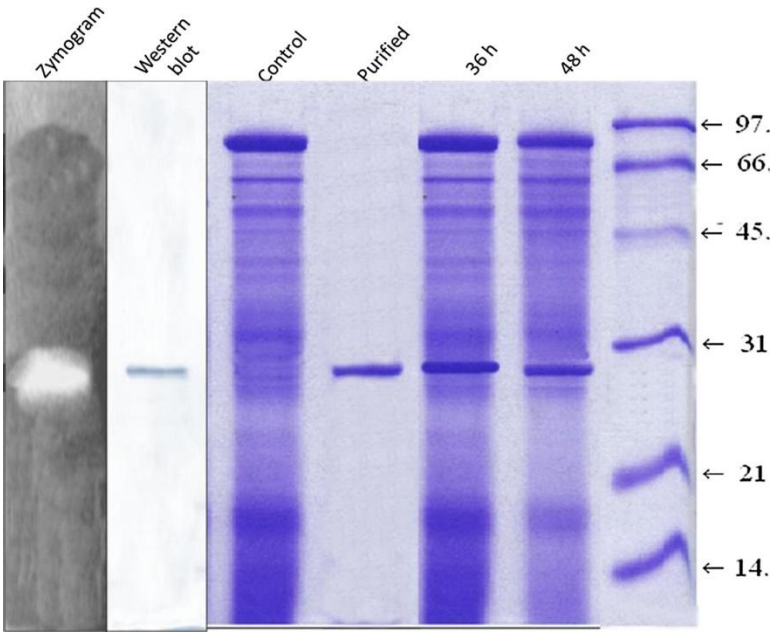


Fig. 3 Western blot, zymogram, and SDS-PAGE analyses confirming the expression and purity of TALipA produced in *Pichia pastoris* X-33

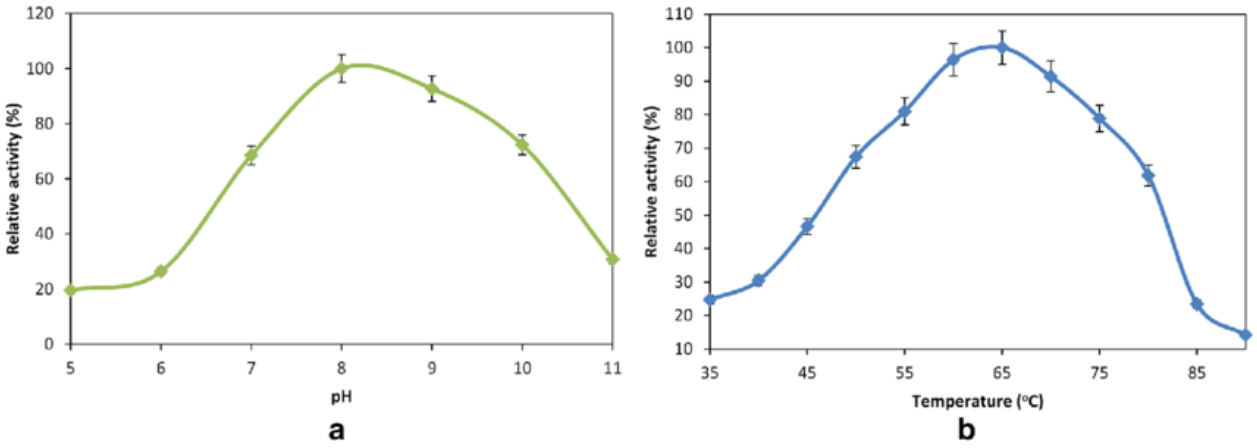


Fig. 4 Temperature (b) and pH (a) effects on TALipA activity. The following buffers were used: glycine–NaOH–NaCl (pH 11), sodium bicarbonate (pH 10), potassium phosphate (pH 8), citrate–phosphate (pH 3–7), and Tris–HCl (pH 9)

SDS-PAGE was used to verify purity, and zymogram and Western blot analysis for His-tag protein were used to confirm it (Fig. 3).

Catalytic Properties

At 60 °C and pH 8.0, the recombinant lipase showed its highest catalytic activity (Fig. 4a, b). The native enzyme, on the other hand, demonstrated an optimum at pH 9.0 and 40 °C [4], most likely as a result of the different protein folding environments in the native and heterologous expression systems [11]. Comparable results have been reported for *Bacillus* lipases, which also display optima at pH 8.0 and 60 °C [12]. Over half of TALipA's activity was maintained over a wide pH range of 6.0 to 10.0 and 30 to 70 °C. The enzyme's alkali tolerance, which is unusual for yeast lipases, which typically work best at acidic to neutral pH values, was demonstrated by the fact that it remained above 50% active even at alkaline pH 10.0 [2]. This unusual property may be related to the conserved AHSMG pentapeptide, which potentially shifts the catalytic center's pKa toward alkaline conditions, unlike the typical GXSXG motif found in most yeast lipases that favors acidic to neutral ranges [13, 14].

At 70 °C, TALipA displayed a half-life of 68 minutes (Table 1), signifying notable thermostability. Such high t½ and D-values are characteristic of heat-stable enzymes, while most yeast lipases are generally mesophilic [7, 15]. The calculated Kd value for TALipA was similar to that was documented for Bacillus subtilis NS8 thermostable lipase [15]. Further amino acid composition analysis supported its thermal stability: TALipA contained fewer uncharged polar residues (17.7%) but a higher proportion of proline residues (7.8%), compared with *B. subtilis* NS8 lipase (25.4% uncharged polar residues, 2.2% proline). Although the arginine/lysine ratio was lower in TALipA, the high proline content may contribute significantly to its stability. These observations indicate that thermostability is not solely determined by amino acid composition but also by overall protein structural features [7, 15].

Table 1 Thermal kinetics

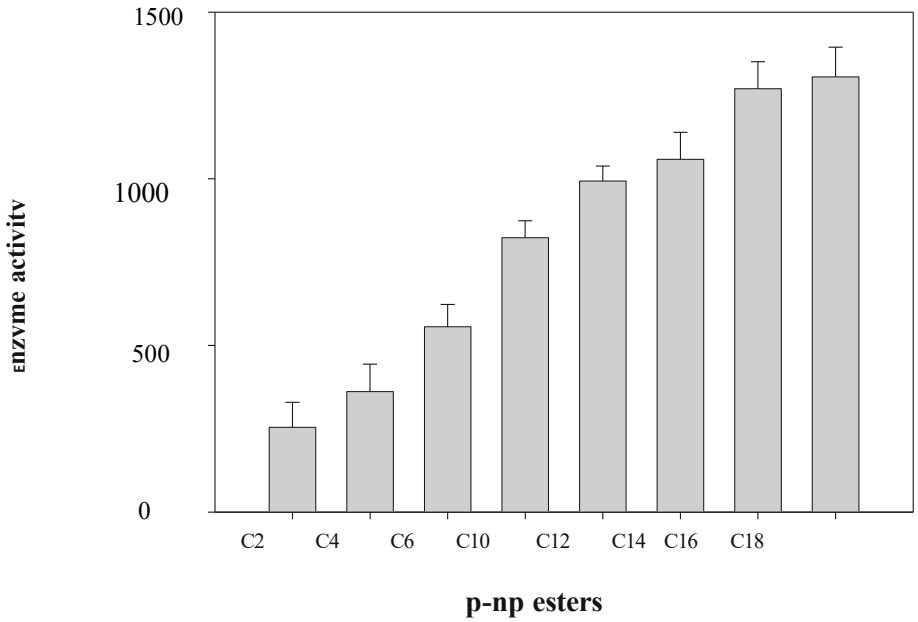
Recombinant lipase	Temperature °C	K_d	$t_{1/2}$ (min)	D-value (min)
TALipA	50	4.2×10^{-3}	165	548
	60	5.6×10^{-3}	123	411
	70	10.1×10^{-3}	68	227

Substrate Specificity and Selectivity

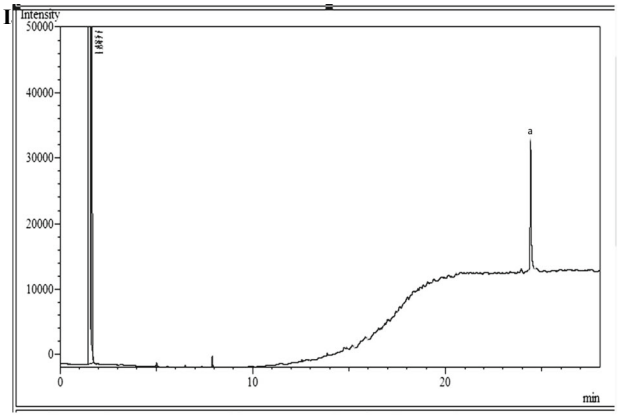
TALipA displayed a strong preference for the strongest activity against p-NP palmitate and stearate is found in long-chain p-NP esters. This pattern was consistent with hydrolysis of a triacylglyceride mixture, where palmitic acid was released in 10 minutes, followed by myristic acid in 30 minutes. Yeast lipases such as *Trichosporon fermentans* and *Geotrichum candidum* exhibit this level of specificity.

Gas chromatography analysis of triolein hydrolysis confirmed that TALipA is regioselective, cleaving preferentially at positions sn-1 and sn-3 to yield oleic acid and mono-/diacylglycerols. This regioselectivity is rare among yeast lipases, which are typically non-regioselective, with exceptions such as *Rhizopus arrhizus*.

a



b



c

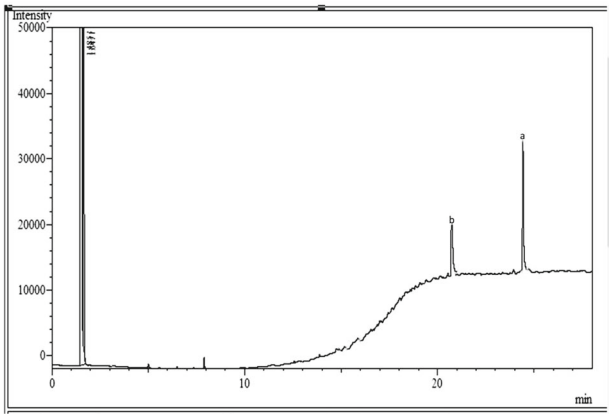


Fig. 5 (a) The substrate specificity of TALipA for p-NP esters with varying chain lengths under optimal assay conditions. (b, c) GC chromatograms of hydrolysis products from an equimolar triacylglyceride mixture after 10 min (b) and 30 min (c), showing peaks corresponding to palmitic and myristic acids..

Enantioselectivity

The enzyme exhibited solvent-dependent enantioselectivity during esterification of racemic phenylethanol with myristic acid. In 1,4-dioxane, the lipase showed S-enantioselectivity (74% ee), while in isopropanol and hexane, the preference shifted to the R-enantiomer, which is 87% and 44% ee, respectively. Such solvent-induced reversal of stereoselectivity suggests conformational flexibility of TALipA, making it a versatile candidate for chiral synthesis.

The regioselective behavior of the lipase was analyzed through GC after hydrolysis of triolein. The appearance of monolein peaks in the chromatogram (Fig. 6) confirmed its regioselective nature. Oleic acid, 1,2(2,3)-diacylglycerols, and 2-monoacylglycerols were released when the enzyme selectively broke down ester bonds at the sn-1 and sn-3 positions. Except for enzymes from *Rhizopus arrhizus* and *Fusarium heterosporum*, the majority of yeast and fungal lipases are known to be non-regioselective [18].

The enantioselectivity of lipases is another important characteristic, which allows them to distinguish between a racemic mixture's enantiomers. In the pharmaceutical industry,

enantiomerically pure or enriched compounds are highly prized, agriculture, synthetic organic chemistry, and natural product synthesis [19]. Using HPLC, the enantioselectivity of TALipA was assessed for the esterification of racemic phenylethanol with myristic acid (Table 2). In 1,3-dioxane (60% v/v), the enzyme favored the S-enantiomer, achieving 74% ee with a 55% conversion rate. Interestingly, when the reaction was carried out in isopropanol or hexane, the preference shifted toward the R-enantiomer, showing 88% and 45% ee, respectively. Enantioselectivity has also been documented in other yeast lipases, including *Candida antarctica, Candida rugosa*, and *Geotrichum candidum* [20]. The solvent-dependent reversal of enantiopreference observed in TALipA is consistent with earlier findings on *T. asahii* MSR54, another lipase from the same strain, and is probably caused by conformational changes in the structure of the enzyme [3, 21]. This feature can be used to create particular stereoselective bioconversions.

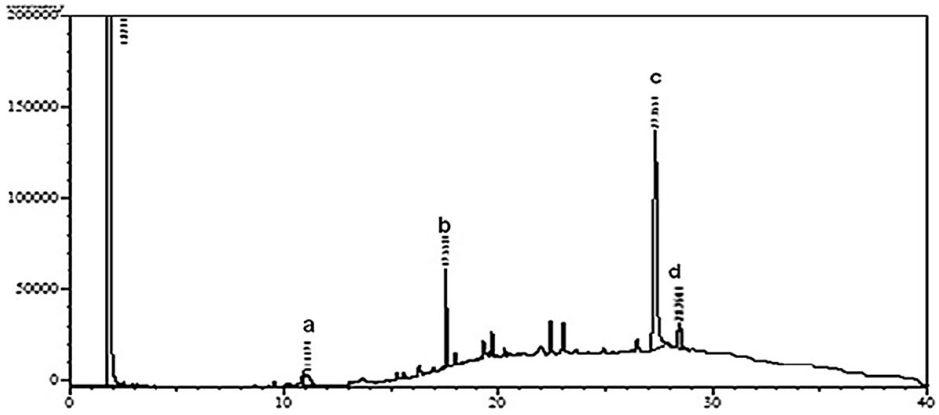


Fig. 6 Regioselectivity of TALipA determined by GC analysis of hydrolysis of triolein in hexane. Monolein, diolein, oleic acid, and triolein are represented by peaks a, b, c, and d, which have respective retention times of 11.1, 17.6, 27.6, and 28.6 minutes.

Table 2 Solvent effects on TALipA's chiral selectivity during racemic phenylethanol esterification

Solvents	Conversion (%)	ee form	ee* (%)
1,4-dioxane	56	S	75
Isopropanol	47	R	88
Hexane	33	R	43

After racemic phenylethanol was esterified with myristic acid, its chiral resolution was examined using HPLC. The resulting chromatograms (Supplementary Fig. 2a and b) were used to calculate

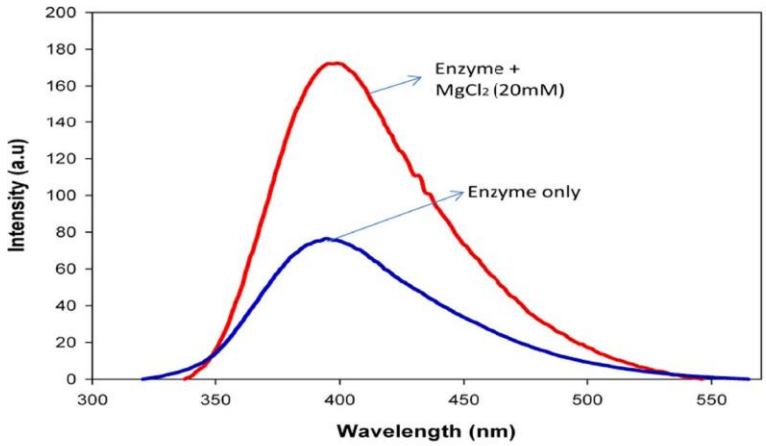
enantiomeric composition. The conversion rate was determined by titrating the unreacted fatty acid with 50 mM NaOH, following the method reported by Kumar et al. (2009) [10].

Impact of Inhibitors, Chelators, and Metal Ions

TALipA activity increased significantly when magnesium ions are present, confirming its nature as a magnesium-activated metalloenzyme. In contrast, Cd^{2+} and Hg^{2+} inhibited the enzyme, while Ca^{2+} and Ba^{2+} had negligible effects. EDTA strongly inhibited activity, which could be reversed upon addition of Mg^{2+}, as confirmed by fluorescence spectroscopy. Inhibitor assays showed sensitivity to PMSF and N-bromosuccinimide, indicating that the enzyme is a serine hydrolase with catalytically important tryptophan residues.

Solvent Stability

TALipA retained stability in a wide range of polar and non-polar solvents. The enzyme remained highly active in DMSO, ethyl acetate, toluene, and hexane, while butanol reduced stability. Solvent tolerance correlated with log P values, similar to other reported yeast lipases.



b

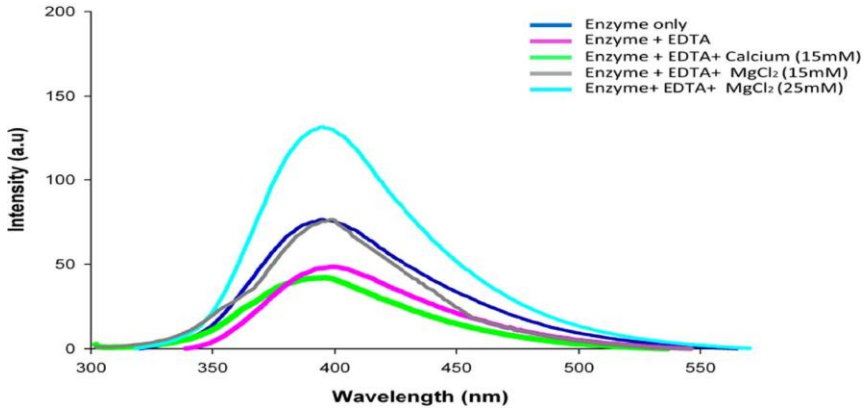


Fig. 7 Intrinsic fluorescence spectra of the enzyme showing activation (a) and restoration of activity in the presence of $MgCl_2$ following EDTA treatment (b).

Subsequently, the influence of different effectors— comprising solvents, chelating agents, inhibitors, and metal ions—was examined. Among the tested ions, magnesium significantly enhanced enzyme activity, showing more than 150% relative activity up to 20 mM. In contrast, cadmium and mercury strongly inhibited activity, whereas calcium and barium had little to no effect up to 11 mM (Fig. S1). This activation caused by magnesium is in line with previous discoveries regarding *Arxula adeninivorans* lipase [22], while the negligible response to calcium resembles the behavior of *Kurtzmanomyces* lipase [23].

Chelating agents such as EDTA and EGTA reduced enzymatic activity, with EDTA having the stronger inhibitory effect (Table S2). Interestingly, inhibition by the addition of 16 mM $MgCl_2$ completely reversed the effects of EDTA (Table S3). Fluorescence spectroscopy provided further evidence of this effect (Fig. 7a and b). In the presence of 20 mM $MgCl_2$, The enzyme's intrinsic fluorescence grew from 76 to 171 AU, indicating structural changes associated with enhanced activity (Fig. 7a). When incubated with 10 mM EDTA, fluorescence dropped to 30 AU, reflecting disruption of Mg^{2+} binding and alteration in protein conformation. Reintroduction of 15 mM $MgCl_2$ restored fluorescence to 76 AU, suggesting that Mg^{2+} binding is essential for maintaining the enzyme's native structure and activity. These effects were not observed with calcium, confirming that TALipA functions as a magnesium-specific metalloenzyme.

Overall Significance

In summary, TALipA is a unique yeast lipase with bacterial-like sequence motifs and unusual biochemical features. It combines thermostability, alkali tolerance, regioselectivity, enantioselectivity, and solvent stability, along with magnesium-dependent activation. These properties make it a promising candidate for industrial biocatalysis, particularly in applications requiring stereospecific transformations.

Conclusion

The 27 kDa lipase gene (TALipA) from *Trichosporon asahii* MSR54 was successfully cloned and expressed in *Pichia pastoris* X-33 in this investigation. Sequence and phylogenetic analyses demonstrated that this lipase is distinct from known yeast lipases, sharing greater similarity with bacterial lipases, and containing unique motifs such as the GL oxyanion hole and the AHSMG pentapeptide. Biochemical characterization revealed that TALipA is thermostable, alkali-tolerant, regioselective, and exhibits solvent-dependent enantioselectivity. Furthermore, it was identified as a magnesium-activated metalloenzyme, a rare feature among yeast lipases.

Collectively, these findings suggest that TALipA is a novel enzyme with several industrially valuable traits. Its stability in diverse solvents, preference for long-chain fatty acids, and ability to switch enantioselectivity depending on solvent conditions make it a promising candidate for applications in pharmaceuticals, fine chemicals, food, and oleochemical industries.

References

1. Gopinath, S.C.B., Anbu, P., Lakshmipriya, T., & Hilda, A. (2013). BioMed Research International, 2013, Article ID 154549, 1–10.
2. Vakhlu, J., & Kour, A. (2006). Electronic Journal of Biotechnology, 9, 69–85.
3. Kumar, S. S., Arora, N., Bhatnagar, R., & Gupta, R. (2009). Journal of Molecular Catalysis B-Enzymatic, 59, 41–46.
4. Kumari, A., & Gupta, R. (2012). Asian Journal of Biotechnology, 4, 70–82.
5. Kumari, A., & Gupta, R. (2013). Journal of Proteins and Proteomics, 4, 5–10.
6. Tuschiya, K., Nakamura, Y., Sakashita, H., & Kimura, T. (1992). Bioscience, Biotechnology, and Biochemistry, 56, 246–250.
7. Kumari, A., Verma, V. V., & Gupta, R. (2012). World Journal of Microbiology and Biotechnology, 28, 3103–3111.
8. Kumari, A., & Gupta, R. (2012). Biotechnology Letters, 34, 1733–1739.
9. Kanjanavas, P., Khuchareontaworn, S., Khawsak, P., Pakpitcharoen, A., Pothivejkul, K., Santiwatanakul, S., Matsui, K., Kajiwara, T., & Chansiri, K. (2010). International Journal of Molecular Science, 29, 3783–3792.
10. Kumari, A., & Gupta, R. (2013). Enzyme Microbial Technology, 53, 386–390.
11. Kademi, A., Lee, B., & Houde, A. (2003). Indian Journal of Biotechnology, 2, 346–355.
12. Quyen, D. T., Dannert, C. S., & Schmid, R. D. (2003). Protein Expression and Purification, 28, 102–110.
13. Ma, J., Zhang, Z., Wang, B., Kong, X., Wang, Y., Cao, S., & Feng, Y. (2006). Protein Expression and Purification, 45, 22–29.
14. El-Shafei, H. A., & Layla, A. (1997). Microbiological Research, 152, 199–208.
15. Olusesan, A. T., Azura, L. K., Forghani, B., Bakar, F. A., Mohamed, A. K. S., Radu, S., Manap, M. Y. A., & And Saar, N. (2011). New Biotechnology, 6, 738–745.
16. Yan, J., Yang, J., Xu, L., & Yan, Y. (2007). Journal of Molecular Catalysis B: Enzyme, 49, 28–35.
17. Fernández, L., Victoria, I. P., Zafra, A., Benítez, P. L., Morales, J. C., Velasco, J., & José, L. A. (2006). Protein Expression and Purification, 49, 256–264.
18. Meghwanshi, G. K., Agarwal, L., Dutt, K., & Saxena, R. K. (2006). Journal of Molecular Catalysis B: Enzyme, 40, 127–131.
19. Cabrera, Z., Fernandez-Lorente, G., Fernandez-Lafuente, R., Palomo, J. M., & Guisan, J. M. (2008). Journal of Molecular Catalysis B: Enzyme, 57, 171–176.
20. Stránský, K., Kejík, M. Z. Z., Macková, Z. W. M., & Demnerová, K. (2007). Biochemical Engineering Journal, 34, 209–216.

21. Othman, S. S., Basri, M., Hussein, M. Z., Rahman, M. B. A., Rahman, R. N. Z. A., Salleh, A. B., & Jasmani, H. (2008). Food Chemistry, 106, 437–443.
22. Boer, E., Mock, P. H., Bode, R., Gellissen, G., & Kunze, G. (2005). Yeast, 22, 523–535.
23. Kakugawa, K., Shobayashi, M., Suzuki, O., & Miyakawa, T. (2002). Bioscience Biotechnology Biochemistry, 66, 978–985.
24. Sangeetha, R., Arulpandi, I., & Geetha, A. (2011). Research Journal of Microbiology, 6, 1–24.
25. Alberta, N. A., Aryee, A., Benjamin, K., Simpson, A., Reynaldo, B., & Villalonga, B. (2007). Enzyme Microbial Technology, 40, 394–402.