JPP JOURNAL OF PROTEINS AND PROTEOMICS
4(1), June 2013, pp. 5-10

Research Article

HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE LIP11 FROM *Yarrowia lipolytica* in *Pichia pastoris* X33

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Abstract: A gene encoding Lip 11 from *Yarrowia lipolytica* MSR80 was cloned and expressed into methanol inducible expression vector pPICZαA and successfully transformed into *Pichia pastoris* X33. The recombinant mut$^+$ clones were selected on zeocine-YPD plates and high-yield clones was identified by tributyrin agar plate screening. The clone produced 32110 U/L of enzyme after 48 h expression in BMMY medium following induction with 0.5% methanol. Lip 11 was purified by affinity chromatography using Ni$^+$ - NTA column with a purification fold of 27 and yield of 59%. It was expressed as glycosylated protein of molecular mass 47 kDa. Biochemical characterization revealed that it was more thermostable with improved K_d value of 5.5 x 10^{-3} at 70 °C and better catalytic efficiency than the previously expressed *E.coli* recombinant enzyme.

Keywords: *Yarrowia lipolytica* MSR80; Lip 11; *Pichia pastoris* X33; *E. coli*

Introduction

Lipases are triacylglycerol hydrolases which have the ability to catalyze hydrolytic reactions in aqueous and synthetic reactions in non aqueous system (Yu *et al.*, 2007). They are invaluable biocatalysts, which can be used in wide range of industrial sectors, be it pharmaceutical or neutraceutical (Ficker *et al.*, 2011).

Lipases are distributed among all living organisms making them ubiquitous. Yeast lipases are highly in demand because of their wide substrate specificity, solvent stability and thermostability (Vkalu *et al.*, 2006). In this respect, *Yarrowia liplolytica* was used for the current study. It is non-conventional oleaginous yeast, generally considered as GRAS organism and well known for its lipolytic nature. It has large diversity of lipases distributed throughout its genome (Ficker *et al.*, 2011). However, till date only Lip 2 has been extensively studied since it was the major extracellular lipase isolated from wild *Yarrowia lipolytica* strain (Ficker *et al.*, 2011). Beside this, nine other lipases from the same organism have been heterologously expressed and biochemically characterised till date (Fiker *et al.*, 2005; Yu *et al.*, 2007a, b; Zaho *et al.*, 2011; Kumari and Gupta 2012a, b).

In our previous study, we had cloned and expressed four lipases - Lip 11, Lip 12, Lip 14 and Lip 18 - from *Yarrowia lipolytica* MSR80 in *E. coli* HB101 pEZZ18 host vector system. Detailed biochemical characterization of those lipases revealed that Lip 11 was closely related to Lip 2 and shared 62% homology with it (Kumar *et al.*, 2012). Among these, Lip 11 had broad range of pH stability and thermostability in comparison to Lip 2. So, it was worthwhile to express it in a suitable yeast system for further studies. For such reason, *Pichia pastoris* is believed to be an ideal system due to higher extracellular yield and

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Received: May 14, 2013
Accepted: June 30, 2013
Published: July 30, 2013

moderate glycosylation of recombinant proteins (Yu *et al*., 2007).

Therefore, here we report cloning and expression of Lip 11 in *P. pastoris* X-33 using pPICZáA as a vector. It was further biochemically characterized and compared with recombinant protein from *E. coli* where it had an additional 14 kDa N-terminal IgG binding domain tag.

Material and Method

Materials - *Yarrowia lipolytica* MSR 80 and *E. coli* DH5α were procured from laboratory culture collection. Chemicals for preparation of potassium phosphate buffers, sodium chloride were purchased from SRL. Plasmid pPICZαA, *P. pastoris* X-33 and Zeocine was from Invitrogen. Gel extraction kit and plasmid isolation kit were from Qiagen. Lipase substrate triacylglycerides, *p*-np esters were procured from Sigma Aldrich USA. Luria bertani, tryptone, yeast extract, yeast nitrogen base and methanol were purchased from Hi-Media, INDIA.

Construction of recombinant plasmid lip11-pPICZαA - The *lip* 11 from *Yarrowia lipolytica* MSR80 were amplified using forward primer 5′ GAATTCAACAGGGTCGTGGAG 3′ and reverse primer 5′ GCGGCCGCAATGGTGCC 3′ having *Eco*RI and *Not*I restriction site. PCR product was cloned in pGEMT easy vector for sequencing. Positive clone was selected by colony PCR and confirmed by sequencing at Central Instrumental Facility (CIF), University of Delhi South Campus, New Delhi. The positive clone was taken to amplify Lip 11 gene with the primers flanking *Eco*RI and *Not*I restriction sites, this was further digested by *Eco*RI and *Not*I, followed by ligation in linearised pPICZαA. The plasmid was then transformed in *E. coli* DH5α. These clones were selected on low salt (0.5% NaCl) LB supplemented with zeocine (25.0 µg/ml) and positive clones were screened with fallout analysis. The DH5α harbouring *lip* 11 were cultured in low salt LB supplemented with same amount of zeocine at 37 °C 200 rpm. The recombinant plasmid was isolated from this over night grown culture by Qiagen maxi prep plasmid isolation kit.

Transformation of recombinant plasmid in P. pastoris X-33 - The recombinant plasmid was first linearized with *Sac*I restriction enzyme and transformed with carrier DNA in *Pichia pastoris* X33 by lithium acetate method according to manufacturer's instruction (Invitrogen). These cells were incubated in 1ml (1M) sorbitol at 30 °C, without shaking for 3.0 h and then transferred to YPDS (1.0% yeast extract (w/v), 2.0% peptone (w/v), 2.0% dextrose (w/v), 1.0 M sorbitol and 1.5% agar) plates having 25.0 µg/ml of zeocine. After 3.0 days of incubation, clones were transferred on YPDS zeocine plates having different concentration of zeocine *viz*; 50.0, 100.0, 200.0, 500.0 µg/ml. The positive clones were further screened on TBA (1.5% tributyrin, 2.0% agar plates) to select the maximum lipase producing clone.

Expression in Pichia pastoris X-33 - *P. pastoris* harbouring *lip* 11 gene was grown in YPD (1.0% yeast extract, 2.0% peptone, 2.0% dextrose) medium till OD_{600} = 10.0 (16.0 h) and then inoculated in BMGY (1.0% yeast extract (w/v), 2.0% peptone (w/v), 1.0% glycerol (w/v), 100.0 mM potassium phosphate buffer pH 7.0, 1.34% yeast nitrogen base (w/v)) medium so that it reaches 0.1 OD_{600} and incubated at 30 °C and 200 rpm till the cell density reached OD_{600} = 7.0. Further cell were pelleted down and resuspended in 50.0 ml BMMY (1.0% (w/v) yeast extract, 2.0% (w/v) peptone, 100.0 mM potassium phosphate buffer pH 7.0, 1.34% (w/v) yeast nitrogen base) medium to obtain cell density OD_{600} = 2.0 and incubated at 30 °C and 200 rpm, methanol (0.5%) was supplemented after 3.0 h of incubation initially, and then after every 24.0 h.

Lipase assay and protein estimation - Enzyme assay was performed using *p*-nitrophenyl palmitate (Gupta *et al.* 2002) and confirmed by titrimetry (Naka and Nakamura, 1992) using 10.0% (v/v) olive oil as substrate. One unit of lipase was defined as the amount of enzyme required to release 1.0 µmole of *p*-nitrophenol or fatty acid, respectively, per ml per min at the optimum pH and temperature. Total protein was estimated by the Bradford method as standard protein.

Enzyme purification - The recombinant lipase was purified by Ni^{2+}- NTA affinity chromatograph. Purification procedure was carried out as per manufacture's instruction with few modifications. The fermentation broth was

subjected to ultra filtration by 10 kDa molecular cut off followed by acetone precipitation in 1:1 ratio. The pellet was dissolved in solution A (50.0 mM phosphate buffer pH 7.5 supplemented with 5.0 mM β-mercapto ethanol). Nickel matrix was charged by using charging buffer *viz*; solution B (solution A + 20.0 mM Imidazole in 50.0 mM phosphate buffer pH 7.5). Then protein was loaded on the matrix. It was left for 1.0 -2.0 h on shaking condition (80 rpm), unbound protein was collected by washing the matrix with 10.0 ml of MQ (milli Q water) followed by washing with 10 ml of solution B. Next, single washing was given with solution C (10 mM sodium phosphate buffer supplemented with 5 mM β- mercaptoethanol and 10% glycerol) and finally elution was done with elution buffer (gradient of imidazole concentration from 50 mM to 200 mM in phosphate buffer pH 7.5 (10 mM)). Purity of the enzyme was checked on SDS-PAGE (Laemmli, 1970). Western blotting was carried out according to Liu *et al.* (2012). Glycosylation of protein was checked by glycosylation kit from G-biosciences according to manufacturer's instruction.

Biochemical characterization - Thermal and pH characterisation, substrate specificity were studied as described earlier (Kumari and Gupta 2012). *E.coli* protein was produced and purified using previously developed method using pEZZ18 clone (Kumari *et al*., 2012) and kinetic constants (K_m and V_{max}) of the recombinant enzymes were determined using *p*-nitro phenol palmitate according to Kumari *et al*., 2012.

Statistical analysis - All experiments were repeated three times in duplicate. Data was plotted with mean ± SD. Mean and SD was calculated using sigma software.

Result and Discussion

Screening and Verification of lipase Producing Clones

The *lip* 11 gene from *Y. lipolytica* MSR 80 (NCBI Bankit under protein I.D AFH77826) was amplified from the recombinant plasmid pEZZ18 having *lip* 11 as insert (Kumari *et al.*, 2012). Amplified product was cloned in pPICZαA and transformed into *P. pastoris* using linearized recombinant *lip* 11-pPICZαA vector by chemical method. The recombinant *lip* 11 was supposed to integrate in to genome of *P. pastoris* by homologous recombination. Selection of recombinant was done on zeocine containing media, which give mut^+ recombinant strains (Krainer *et al.*, 2012) followed by screening on TBA plate for functional expression of lipase. Strain mut^+ was characterised as *P.pastoris* having wild type *AOX*1 and *AOX*2, which can metabolize methanol at faster rate (Krainer *et al.*, 2012). Clone number four with highest zone of hydrolysis was considered for further studies Fig. 1. Integration of lipase gene was confirmed by PCR using gene specific primer on the genomic DNA of lipase positive clone. A band corresponding to 1.2 kb showed that *lip* 11 was successfully integrated to the yeast genome. Recombinant clones could tolerate upto 200 µg/ml of zeocin concentration suggesting that there was only one copy insertion of the recombinant gene (Ali *et al.*, 2011). Lipase activity in the positive recombinant after methanol induction confirmed the production of lipase under *AOX*1 promoter.

Lipase Purification and Comparative Biochemical Characterization

Lipase production was carried out in 250 ml shake flask as described above and cells were harvested after 48 h of methanol induction. Cell free supernatant was analyzed for lipase activity showing total activity of 32110 U/L which was 100 times higher than that of Lip 11 produced in *E. coli* (Kumari *et al.*, 2012). This in confirmation with the fact that *P. pastoris* system is more efficient secretary system than *E. coli*.

The recombinant lipase protein was purified by affinity chromatography with the purification fold of 2.7 and 59% yield. Purity of enzyme was checked by SDS-PAGE analysis and confirmed by western blot analysis for His-tag protein (Fig. 2). There was a 3kDa shift in the molecular weight of the protein, when expressed in *Pichia pastoris* due to glycosylation confirmed by staining method (Fig. 2 c).

It was characterized and compared with that of Lip11 produced in pEZZ18 harbored in *E. coli* HB101 (Kumari and Gupta 2012). Recombinant Lip11 produced in *E. coli* and *P. pastoris* has more or less similar properties like pH, temperature

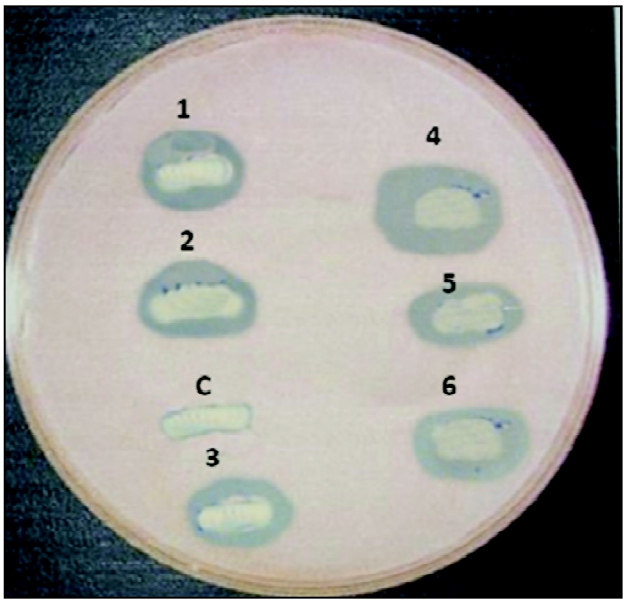


Figure 1: **Screening of *Pichia pastoris* X33 harbouring Lip11 on TBA plate**
* out of 60 clones only best six clones are shown in the figure

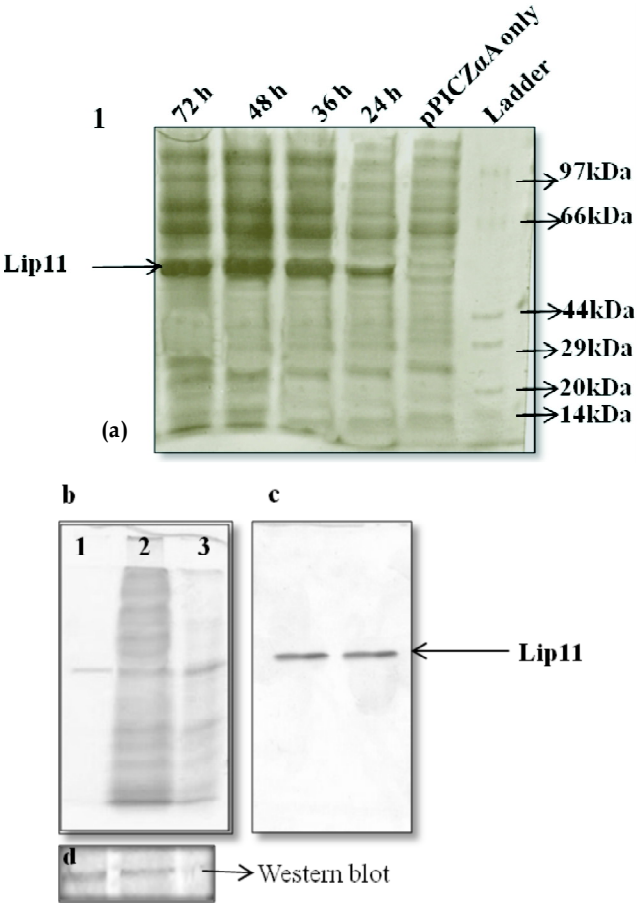
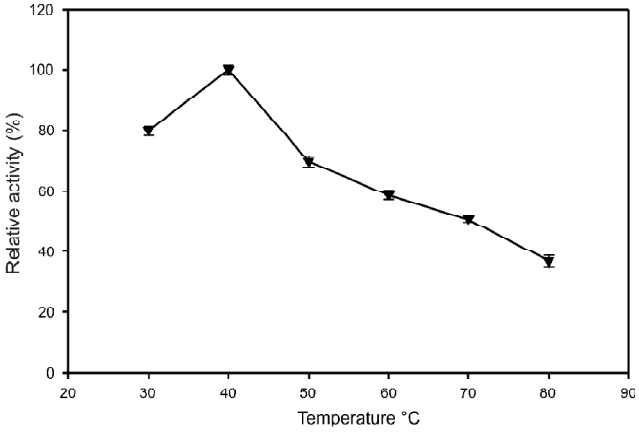


Figure 2a: **Time profiling of Lip11 in *Pichia pastoris*. b. SDS-PAGE analysis of purified lipase: On 10 % SDS – PAGE lane 1 = purified protein, lane 2 = acetone precipitated protein and lane 3 = crude protein. c. glycosylation stained gel and d. Western blot analysis.**

optima (Fig 3a, b). It also showed broad range of substrate specificity on triaclyglycerides, *p*-np-esters and oils keeping similar trends in the substrate specificity as in *E. coli* for the mid to long chain length substrate (Fig. 4). However, Lip11 produced in *P. pastoris* was more thermostable than that of in *E. coli* with $t_{1/2}$ of 266 min and 126 min at 40 °C and 70 °C respectively (Table 2). This may be due to the fact that proteins produced by yeast are glycosylated (Daly and Hearn 2004).

The recombinant enzyme from *P.pastoris* was catalytically more efficient with lower K_m and higher V_{max} than that from recombinant *E.coli*. The Kinetic parameters K_m and V_{max} of Lip 11 were 1.67 µM, 1.0 mM/min and 2.5 µM, 0.8 mM/min for *P. pastoris* and *E. coli* respectively for the hydrolysis



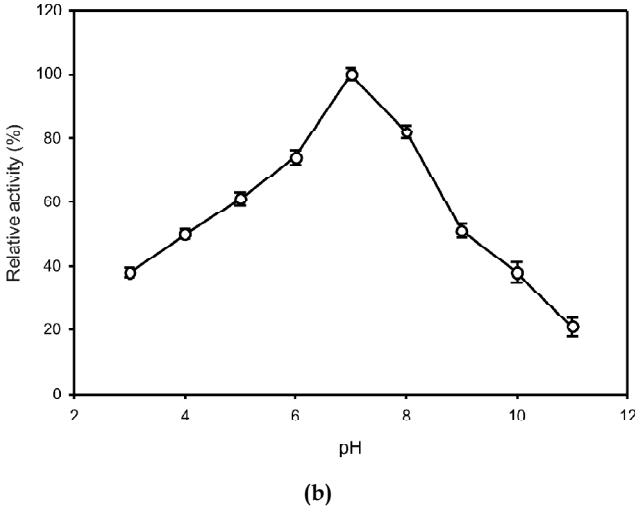


Figure 3a: **Per cent relative enzyme activity as a function of temperature* at pH 7.0 b. Percentage relative enzyme activity as a function of pH* at temperature 40 °C.**
* 100% activity corresponds to 3.5 U/ ml protein on *p*-np palmitate at pH 7.0 and 40 °C

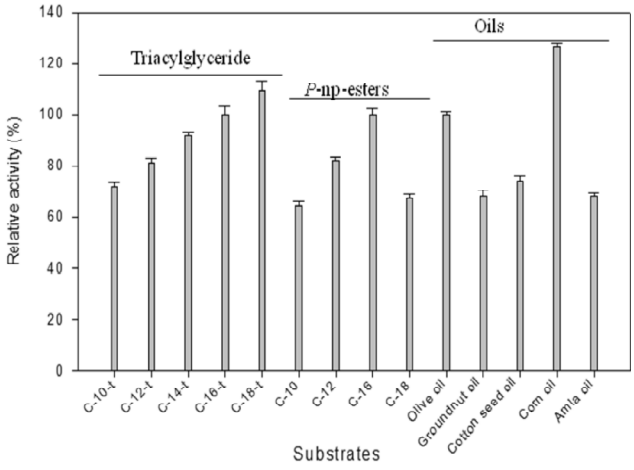


Figure 4: **Specificity of Lip 11 on different substrates**
Where 100 % activity on tripalmitate, *p*-np palmitate and olive oil corresponds to 9.0 U/ml, 3.5 U/ml and 32.0 U/ml respectively.

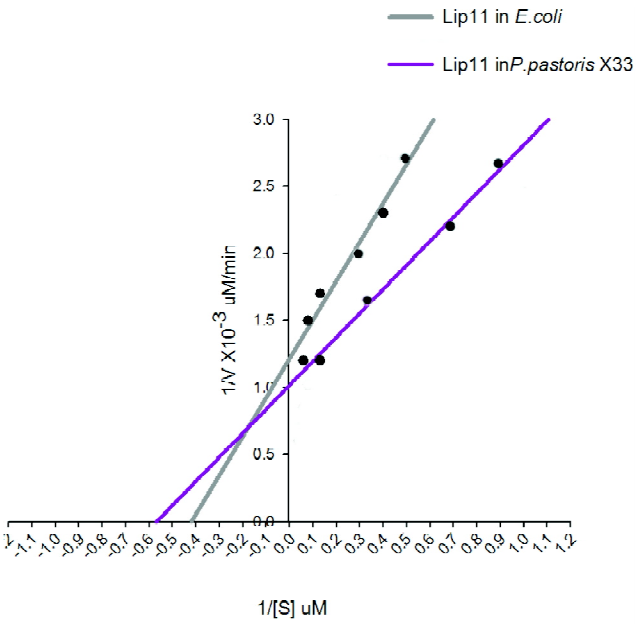


Figure 5: **Lineweaver-Burk plot for Lip11 expressed in *E.coli* and *P. pastoris* X33 using *p*-np palmitate as substrate**

Table 1
Purification Scheme

Purification method	*Total activity (U)*	*Total protein (g)*	*Specific activity (U/mg)*	*Fold purification*	*Yield %*
Crude	32110	2.3	13.9	1	100.0
Acetone precipitation	23142	1.3	17.8	1.3	72.0
Affinity chromatography	18945	0.05	378	27.0	59.0

Table 2
Comparative thermal kinetics of Lip 11 expressed in E.coli and P.pastoris

Kinetics Parameters	*Lip11 in E.coli (Kumari et al., 2012)*	*Lip 11 in P.pastoris (present study)*
at 40°C		
K_d x 10^{-3}	4.2	2.6
$t_{1/2}$ (min)	165	266
D-value (min)	548	885
at 70°C		
K_d x 10^{-3}	13.9	5.5
$t_{1/2}$ (min)	49	126
D-value (min)	165	418

of *p*-nitro phenol palmitate (Fig. 5). This may be because of change in the folding pattern of protein in *E. coli* and *Pichia*. Also in pEZZ 18 *E. coli* system Lip 11 was expressed as fusion protein with 14 kDa IgG binding domain, which may also change the folding pattern of protein and hence affinity for the respective substrate.

In nut shell, *P. pastoris* is not only the efficient system for production of Lip 11, but the recombinant enzyme also exhibited better thermostability and catalytic efficiency than that of *E. coli*.

Acknowledgement

Authors acknowledge Council of Scientific and Industrial Research (CSIR) and R & D grant from Delhi University India, for the financial assistance. Arti Kumari acknowledges CSIR for providing senior research fellowship.

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