J Ind Microbiol Biotechnol (2009) 36:427–432
DOI 10.1007/s10295-008-0513-8

ORIGINAL PAPER

A thiol-activated lipase from *Trichosporon asahii* MSR 54: detergent compatibility and presoak formulation for oil removal from soiled cloth at ambient temperature

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Received: 6 September 2008 / Accepted: 4 December 2008 / Published online: 23 January 2009
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Abstract An alkaline lipase from *Trichosporon asahii* MSR 54 was used to develop presoak formulation for removing oil stains at ambient temperature. The lipase was produced in a reactor followed by concentration by ultrafiltration and then it was dried with starch. The biochemical characteristics of enzyme showed that it was an alkaline lipase having pH activity in the range of pH 8.0–10.0 and temperature in the range of 25–50°C. The present lipase was active >80% at 25°C. The lipase was cystein activated with fourfold enhancement in presence of 5 mM cystein and likewise the activity was also stimulated in presence of papain hydrolysate which served as source of cystein. The presoak formulation consisted of two components A and B, component A was enzyme additive and B was a mixture of carbonate/bicarbonate source of alkali and papain hydrolysate as source of cystein. The results indicated that the presoaking in enzyme formulation followed by detergent washing was a better strategy for stain removal than direct washing with detergent in presence of lipase. Further, it was observed that 0.25% presoak component B in presence of 100 U enzyme component A (0.1 g) was the best formulation in removing maximum stain from mustard oil/triolein soiled clothes as indicated by increase in reflectance which was found equal to that of control cloth. The lipase action in presoaked formulation was clearly indicated by quantitated fatty acid release and also the TLC results of wash water, where oil hydrolytic products were visible only in presence of enzyme in the treatment. The wash performance carried at 25°C indicated that washing at 25°C was at par with that at 40°C as indicated by similar reflectance of the washed cloth piece though qualitative fatty acid release was higher at 40°C.

Keywords Detergent · *Trichosporon asahii* · Presoak formulation · Wash performance analysis · Thiol-activated lipase

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Introduction

Microbial lipases find immense applications in various sectors such as pharmaceuticals, pesticides, detergent, food, oil and leather industry [19]. At present, it finds largest application in detergent industry. The alkaline lipases which show detergent compatibility are preferred as detergent additives for removing oil stains [6, 19]. Generally the washing temperatures are 50–60°C therefore, alkaline thermostable lipases are preferred [14]. However, the present demand for washing at ambient temperature has necessitated the search for new alkaline lipases which are active at low temperatures. In this respect, yeast lipases with alkaline nature are preferred as these generally work at lower temperatures as compared to bacterial and fungal lipases [1, 5, 13]. Here, we report alkaline lipase from *Trichosporon asahii* MSR 54 which was previously isolated from petroleum sludge for its application in developing presoak formulation for oil stain removal at ambient temperatures.

Materials and methods

Chemicals

p-Nitrophenyl palmitate (*p*-NPP) for lipase assay was purchased from Sigma (St Louis, USA). Tweens and Triton X-100, papain hydrolysate from ICN Biomedicals Inc., USA. All other analytical reagents and media components were purchased from Hi-Media, Qualigens or SRL, India. Commercial detergents were obtained locally. All the experiments were performed in triplicate and repeated twice.

Yeast strain and production medium

Trichosporon asahii MSR 54 has been deposited in Microbial Type Cultural Collection and Gene Bank (MTCC), India with accession no. MTCC 9450. Lipase was produced in 14 L bioreactor with 10 L working volume, a medium containing (g/l): yeast extract 20, malt extract 6, glucose 5, KH_2PO_4 1, K_2HPO_4 3, Corn oil 10, casein 4, and $MgCl_2$ 1 (pH 7.0).The production medium was inoculated with 4% of 24-h-old seed culture and incubated at 30°C; 250 rpm in a Chemap AG fermentor, Switzerland for 32 h. The culture not comes into GRAS Status therefore, the cell free enzyme supernatant was obtained by centrifugation at $14{,}000\times g$ for 20 min at 4°C (Sorvall® RC 5C Plus). The crude enzyme preparation was subjected to ultra filtration with 10 KDa membrane (Sartorius, Germany). The retentate solution was further concentrated and dried using soluble starch. This preparation was named as presoak component A.

Lipase assay

Lipase activity in the culture filtrate was determined by *p*-NPP assay [2, 20] and was confirmed by titrimetry [7] using 1% (v/v) olive oil as substrate. One international unit of lipase was defined as the amount of enzyme required to release 1 µmole of *p*-nitrophenol or fatty acid, respectively, per ml per minute at 40°C and pH 9.

Characterization of the enzyme

Effect of pH temperature

Enzyme assay was carried out at different pH (2.0–10.0) and temperature (25–80°C) and relative activity was determined. The enzyme was assayed with buffers of different pH (2.0–10.0) (0.05 M) Phosphoric acid–KH_2PO_4 buffer (pH 2.0), citrate–phosphate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 7.0–8.0), Tris–HCl (pH 9.0), glycine–NaOH (pH 10.0) at 40°C.

Effect of thiols

During biochemical characterization of the enzyme it was observed that activity is enhanced in presence of thiol agents, mercaptoethanol, DTT and cystein. Here, we have reported only the effect of cystein and also papain hydrolysate (a source of cystein) which is to be used in formulation. Effect of cystein (5 mM) and papain hydrolysate (1%) was studied on triolein hydrolysis by *T. asahii* lipase component A by performing titrimetry assay using 1% triolein emulsion with or without cystein at pH 9.0 carbonate/bicarbonate buffer at 25°C.

Effect of oxidizing agents

The enzyme was incubated with different concentrations of oxidizing agents at ambient temperature (25°C) for 1 h and enzyme stability was calculated as residual activity.

Effect of surfactants and detergents on enzyme stability

The enzyme sample was incubated with different ionic and non-ionic detergents and surfactants viz SDS, saponin, tweens, bile salt, and different commercial detergents like Ariel, Ezee, Rin supreme, Surf-ultra, Tide and 555 (1% w/v) for 1 h at ambient temperature and enzyme was assayed for residual activity.

Presoak formulation

The presoak formulation was designed based on the thiol activation and alkaline property of the enzyme. The presoak formulation was prepared as A and B components. A contained enzyme and B was salts. The detailed composition for 100 g of B component is, 36 g Na_2CO_3, 4 g $NaHCO_3$, 20 g papain hydrolysate and 40 g binder (soluble starch) and A component was dry enzyme powder having 1,000 U lipase/g dry powder. Three control experiments were also set: (1) only component B (C1), (2) component A and B with detergent (tide) (C2), (3) only detergent (C3).

Wash performance

Small square piece (5×5 cm^2) of a new cotton fabric was taken. Samples (0.1 ml) of mustard oil and triolein, were used to stain the fabric. The stained cloth was dried and subjected to presoak treatments with varying A and B components according to the experiment in 25 ml tap water for 30 min at 100 rpm. After the prewash treatment 5 ml sample was removed for wash water analysis and then 0.25% tide detergents was added and kept for 30 min washing. Cloth was removed and washed in tap water and dried and used for reflectance studies using on a reflectance meter

(Model no. UEC-1018, Universal Engg Corporation, India).

Wash water analysis

Quantification of release of fatty acid

The wash water was titrated against 0.1 N NaOH using as pH probe. Control (C1) values without enzyme, were subtracted to obtain the fatty acid released in different treatments.

TLC detection of hydrolytic products

The hydrolytic products in wash water were detected by thin layer chromatography (TLC) using hexane:diethyl ether:acetate in the ratio of 80:30:1 as solvent. The spot detection was done by iodine chamber.

Results and discussion

The lipase was produced in a 14 L reactor with 10 L working volume in a corn oil medium. It was observed that maximum lipase 110 U/ml was produced after 32 h which is similar to the lipase production in shake flask studies as reported earlier [16]. The enzyme was concentrated by ultrafiltration and dried with starch to develop dry formulation. The present enzyme is an alkaline lipase active at ambient temperature. The pH range was from pH 8.0 to 10.0 and temperature range from 25 to 50°C with maximal activity at 40°C and more than 80% activity at ambient temperature (25°C) (Fig. 1). This indicates that, enzyme may be suitable for detergent application at ambient temperature. Detergent enzyme which is active at room temperature is desired as present day washing at low temperature is preferred for maintaining the fabric quality [8]. Besides temperature and pH properties the enzyme for detergent application must also be compatible with detergent components like surfactant and bleaches. The detergent compatibility with commercial detergent components (ionic and non-ionic) was studied and it was found to be stable in presence of most of the detergents (1% w/v) after 1 h incubation (Table 1). Further, the enzyme was also stable in presence of oxidizing agents as sodium perborate and hypochlorite with more than 80% residual active after 1 h incubation. It is suggested that, the present enzyme is reasonably bleach stable and can be used as additive to detergents. Bleach stability is one of the desired features for detergent compatibility of enzymes. For maintaining

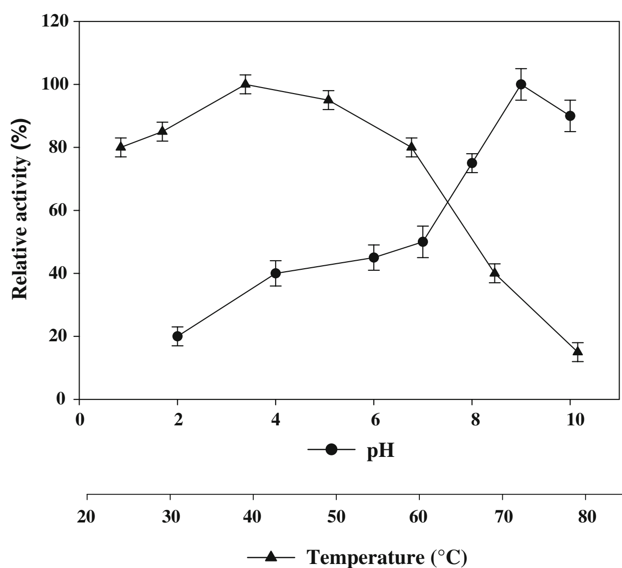


Fig. 1 Effect of different pH and temperature on lipase activity from *Trichosporon asahii* MSR 54

Table 1 Effect of oxidizing agents, surfactants, protease and detergents on relative stability of lipase from *T.asahii* MSR 54

Oxidizing agents, surfactants, protease and detergents (1% w/v or v/v)	*Trichosporon asahii* MSR 54 lipase Residual activity (%)	Residual Enzyme activity (U/ml)
Oxidizing agents		
Sodium perborate (1%)	65	48.8
Sodium hypochlorite (1%)	85	63.7
SDS (1%)	2.2	1.60
H_2O_2 (1%)	67	50.2
Surfactants		
Tween-20	100	75.0
Tween-40	90	67.5
Tween-60	80	60.0
Tween-80	80	60.0
Triton X-100	85	63.7
Saponin	60	45.0
Sodium cholate	95	71.3
Sodium tarocholate	50	37.5
Protease		
Savinase (Novozymes)	25	18.8
Subtilisin (Sigma)	5	3.75
Bacillus licheniformis (lab isolate)	15	11.2
Detergents		
Ezee	40	30.0
Ariel	25	18.7
Tide	26	19.5
555	26	19.5
Surf-ultra	27	20.2
Rin Supreme	42	31.5

Standard deviation never exceeded 2%, control activity is 75 U/ml at 40°C and pH 9.0

whiteness of fabric most of the detergents available are with bleaches. In this respect, bleach stable proteases are well known [4, 9, 18]. However, very few lipases are known to have this features [3]. The *T. asahii* lipase also was evaluated for stability in presence of commercial detergent powders available in local market. It was observed that the enzyme was not much stable in any of the commercial detergents. The loss of stability may be because of its sensitivity to other additives present in them. Further, the enzyme was also not stable in presence of commercial proteases which are one of the components of high grade detergents (Table 1). In absence of these properties in the present lipase an attempt was made to develop prewash formulation to clean oil stains. Majority of the literature reports are related to enzyme as detergent additives [10, 11] rather than prewash formulations. However, there are reports of sequential washing regime, where lipolytic activity is carried out at low pH followed by detergent washing at alkaline pH [15].

The present prewash formulation was developed based on the enzyme properties choosing the component among enzyme enhancers so that enzyme consumption can be reduced. The *T. asahii* lipase is an alkaline and thiol-activated lipase and its activity becomes fourfold in presence of 5 mM cystein (Fig. 2). The papain hydrolysate was selected as cystein sources and it was observed that in presence of 1% papain hydrolysate there were twofold enhancements in lipase activity. In addition carbonate–bicarbonate was used as an alkali source since they are cheap and easily available. During presoaking the soiled cloth was soaked for 30 min in presoak formulation, followed by addition of detergent to the same water for washing. The washing was done both at 25 and 40°C using triolein and mustard oil sources of oil stain. Wash performance of presoak formulation in removing oil was tested by analysis of wash water for fatty acid release and also by measuring reflectance of the washed cloth. The wash water analysis and reflectance of fabric as test parameter for wash performance analysis has been used earlier during studies with proteases [12]. The wash water analysis (Fig. 3) of present formulation indicate that in presence of constant enzyme (100 U) fatty acid release and increase in reflectance of the cloth was a function of component B. This is because of the facts that increase in the component B, there was corresponding increase in cystein an enhancer for lipase activity (Fig. 2). Wash performance as a function of enzyme concentration in presence of 0.25% component B shows that up to 20 U there was a steep increase in release of fatty acids. Thereafter, the release of fatty acids was a linear function of enzyme concentration with maximum fatty acid release

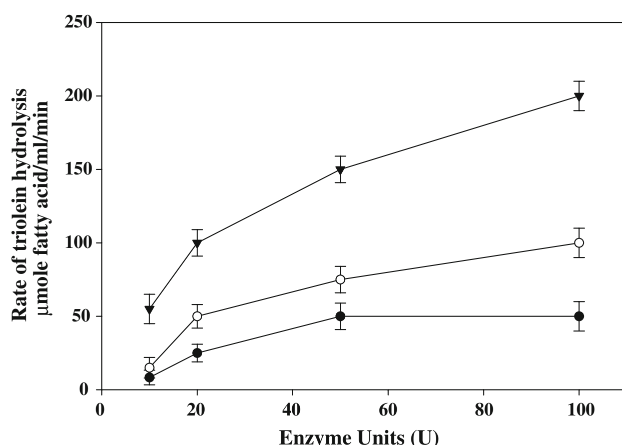
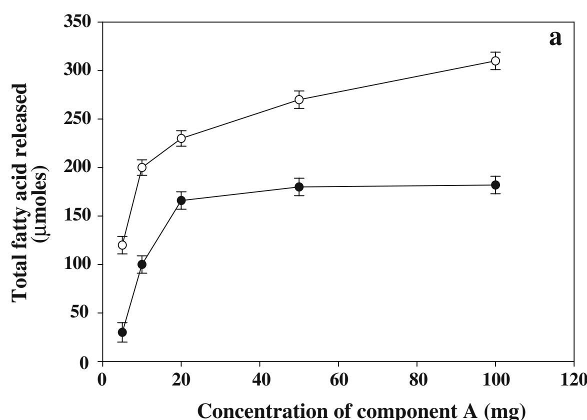


Fig. 2 Effect of cystein (*filled triangles*), papain hydrolysate (*open circles*) and control (*filled circles*) on rate of triolein hydrolysis by *T. asahii* lipase as a function of enzyme concentration at 25°C, pH 9.0



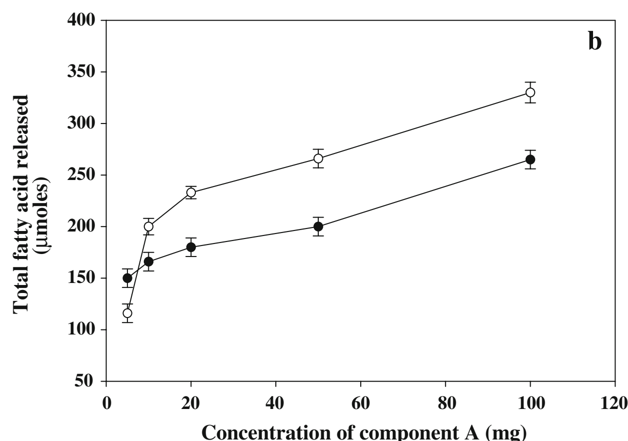
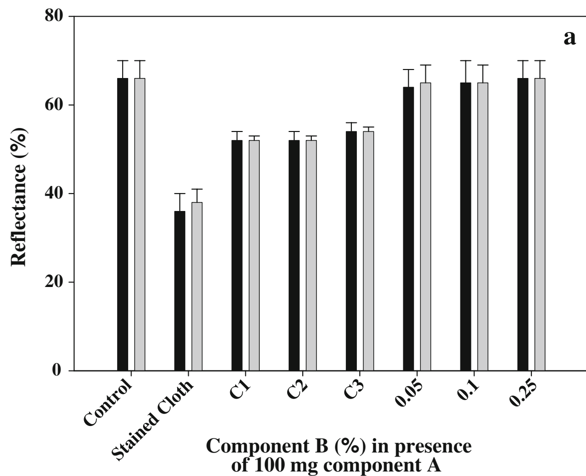


Fig. 3 Effect of varying concentration of component A (enzyme powder) in presence of 0.25% component B of presoak formulation on wash performance of mustard oil (*filled circles*) and triolein (*open circles*) soiled cloth at 25°C (**a**) and 40°C (**b**)



C1: Component B (0.25% w/v) only, C2: Component B (0.25 % w/v) with detergent (1%), C3: Component A (0.1 gm) +B (0.25 % w/v) + detergent (1 %)

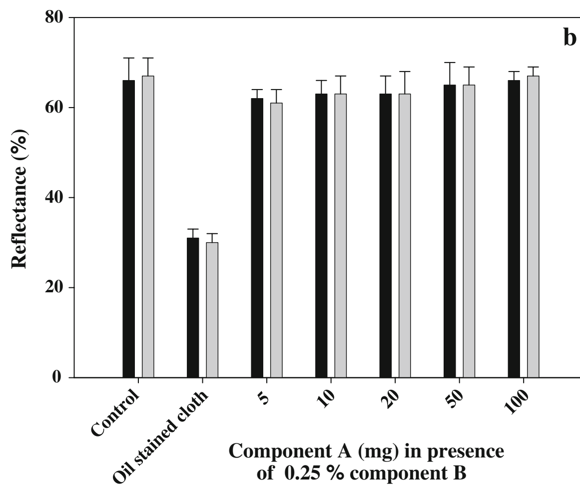


Fig. 4 Effect of presoak formulation components on wash performance as percentage reflectance of triolein (*filled bars*) and mustard oil (*shaded bars*) soiled cloth after washing at 25°C, **a** as a function of component B and **b** as a function of component A

from mustard oil in presence of 50 U and from triolein, 100 U at 40°C, at 25°C 100 U gave best result in both the cases. However, there was not much change in the reflectance at both the temperatures (Fig. 4). In all the enzyme concentration there was significant increase in reflectance approaching near the unstained control in 50–100 U enzyme concentrations.

The lipase action on oil stain was also confirmed by TLC analysis of wash water for hydrolytic products. From the figure (Fig. 5), it can be observed that oil hydrolytic products, were detected in wash water after the presoak treatment (lane 4). However, there exist alternate methods of detecting oil removal from soiled fabric by extracting residual oil from the cloth [17]. However, the method seems not

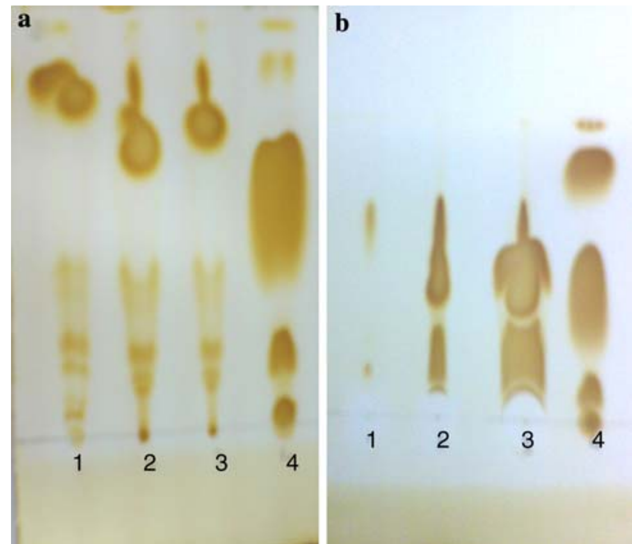


Fig. 5 Qualitative analysis by TLC of wash water after presoaking the stained cloth at 25°C and 100 rpm for 30 min **a** mustard oil and **b** Triolein. *Lane 1* component B (0.25% w/v) only (C1). *Lane 2* component B (0.25% w/v) with detergent (1%) (C2). *Lane 3* component A (0.1 gm) + B (0.25% w/v) + detergent (1%) (C3). *Lane 4* test sample component A (0.1 g) + B (0.25% w/v)

only to be tedious but also error prone as it is a gravimetric method.

Conclusion

Thus, it can be put forth that *T. asahii* MSR 54 lipase can be a good candidate for developing presoak formulation which can be used along with any available commercial detergents at ambient washing temperatures. Finally, the presoak formulation serve as an alternate strategy to enzymes as detergent additives for exploiting those enzymes which are cold adapted, alkaline but do not posses good detergent compatibility. Deoiling spray formulation can be prepared for cleaning oil spots at ambient temperature from variety of surfaces.

Acknowledgments The authors thank Council of Scientific and Industrial Research, New Delhi, for financial assistance for a project on novel yeast lipase [sanction No. 38(1118)/06/EMR-II] and also miscellaneous R&D grant from University of Delhi, India.

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