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A novel halophilic extracellular lipase with both hydrolytic and synthetic activities

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ABSTRACT

Fungal lipases with an ability to catalyze both hydrolytic and synthetic activities are now becoming extremely useful for various industrial applications. However, identification and selection of fungus with this dual ability is challenging. In this direction, we attempted to screen and identify potential marine fungus to produce a novel extracellular lipase using multi-step screening approach. Initially, we qualitatively screened 10 strains isolated from sea water using tributyrin agar plate assay method and six potential producers (SW1 to SW6) were selected which showed halos greater than minimum threshold (3 mm). Further, we quantitatively screened for the hydrolytic activities of crude extracellular lipase and evaluated their synthetic activities (esterification and transesterification reactions) also. The result analysis showed that among the six potential strains, the extracellular lipase produced by SW4 strain exhibited the highest activity. Thus, the selected SW4 strain produced a novel extracellular lipase with a good correlation between hydrolytic and synthetic activities as compared to other strains. Further, the selected strain (SW4) was taxonomically identified as *Fusarium solani* and was deposited in NFCCI under accession No. 4048 and the nucleotide sequence was also deposited in GenBank under accession No. MF138865.

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) belong to the class of serine hydrolases that catalyze synthesis of esters from glycerol and long chain fatty acids as well as hydrolysis of carboxylic ester bonds of triglycerides. The reaction occurs at the interface between insoluble substrate phase and aqueous phase to yield diglycerides, monoglycerides, fatty acids, and glycerol and that they do not require any cofactor for catalysis (Prazeres et al., 2006; Hasan et al., 2006; Babu and Rao, 2007; Ghosh et al., 1996). Lipases are essential and have emerged as leading biocatalysts because of wide range of activities including chemo-, regio- and enantio-selective hydrolyses and in the syntheses of a broad range of compounds through reactions such as esterification, inter-esterification, trans-esterification, alcoholysis, acidolysis, and aminolysis (Jaeger and Eggert, 2002; Savitha et al., 2007). These reactions make it highly potential in industrial applications such as additives in foods, pharmaceuticals, medical assay, cosmetics, leather, dairy industry, fine chemicals, detergents, paper manufacture, waste-water treatment, synthesis of biopolymer and biodiesel, production of agrochemicals and flavors (Hasan et al., 2006; Haki and Rakshit, 2003). These myriad multifaceted applications of lipases have influenced economy, contributing to multibillion dollar in terms of sales after proteases and amylases (Haki and Rakshit, 2003; Rigo et al., 2010).

Lipases are widely distributed in nature and have been found in many species of animals, plants, bacteria, yeasts and fungi and such lipases from different origins differ widely in physical and biochemical properties (Saeed et al., 2005). Among these, microbial lipases have shown high utility compared to other sources because of variety of catalytic activities, high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations, rapid growth of microorganisms on inexpensive media and broad spectrum of industrial application due to their greater stability and substrate specificity (Musa and Adebayo-Tayo, 2012). Among the microorganisms, filamentous fungi are considered as the most important and are currently the preferred sources for industrial applications because the enzyme is usually produced extracellularly and thus facilitating extraction from the fermentation media (Carvalho et al., 2006; Basheer et al., 2011).

The ability of microorganisms, particularly fungi, to produce extracellular enzymes such as lipases is of great survival value in a wide range of inhospitable environmental conditions (Gopinath et al., 2005). Therefore, these are able to produce lipases in several habitats, including wastes of vegetable oil and dairy product industries, soil contaminated with oil, seeds, deteriorated food and marine habitat

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(Sharma et al., 2001). Further, marine fungi are considered as having great potential and efficient producers of biologically and chemically active novel compounds (Trischman et al., 1994). Therefore, in recent years, marine source is being explored for potential new organisms, including those producing lipases (Louhasakul et al., 2016; Anwesha and Bhaskara, 2016). However, there has been lack of focus in screening/selection of marine fungal lipases with an ability to catalyze both hydrolytic and synthetic reactions. Hence, owing to the demand of fungal lipases with unusual features, our approach in this study focused to isolate and screen out wild marine fungal strain with innate ability to produce new and versatile lipase which catalyzes both hydrolytic and synthetic reactions.

2. Materials and methods

2.1. Chemicals

Tributyrin, olive oil, Rhodamine-B, para nitrophenol palmitate, oleic acid (extra pure), sodium hydroxide, *n*-heptane, Bradford reagent, potassium hydroxide and streptomycin were obtained from Himedia.

2.2. Isolation of fungus

The location selected for the collection of marine water is Udupi coastal area of the Arabian Sea in Karnataka state, India. The fungal strains were obtained by using selective medium consisting of (% m/v in sea water) 0.5% peptone, 0.7% beef extract, 0.4% sodium chloride, 2% olive oil at pH 8.5 and fortified with streptomycin to inhibit bacterial growth. After 96 h of incubation, serial dilution of the culture was carried out and aliquots (0.1 ml) were surface plated on tributyrin agar plates (Olutiola et al., 2000). The growing colonies were isolated into Petri dishes containing potato dextrose agar (PDA) medium and were stored at −4 °C.

2.3. Qualitative screening of lipase

2.3.1. Screening of Lipolytic fungi using tributyrin

The screening of isolated fungi for lipolytic activity was performed in Petri dishes using medium constituted by (% m/v in sea water) 0.5% peptone, 0.7% beef extract, 0.4% sodium chloride, 0.2% tributyrin, 2% agar at pH 8.0 (Freire, 1996; Griebeler et al., 2011). The plates inoculated with fungi were incubated at 30 °C for 7 days, and diameter (d) of the colonies and the diameter (D) of total clear lipolytic halos including the colonies were measured. The fungal strains that displayed higher halos (D − d) were selected as promising fungal lipase producers using tributyrin as substrate.

2.3.2. Screening of Lipolytic fungus using Rhodamine B olive oil

The isolated fungi screened for lipolytic activity using tributyrin were further screened in Petri dishes using medium constituted by (% m/v in sea water) 0.5% peptone, 0.7% beef extract, 0.4% sodium chloride, 2% olive oil, 2% agar, and 1 ml of Rhodamine B (1 mg/ml) at pH 8.0 (Kouker and Jaeger, 1987). The medium was shaken thoroughly and spread onto Petri dishes; fungal strains were inoculated and were incubated at 30 °C for 7 days. The production of extracellular lipase enzyme was identified as an orange fluorescent halo under UV light at 350 nm.

2.4. Fungi preparation and extracellular lipase production

The selected fungal strains SW1 through SW6 were screened using agar plate method and confirmed subsequently using Rhodamine B olive oil plate for the true production of extracellular lipase. The fermentation was carried out in 250 ml Erlenmeyer shake flasks using medium constituted by (% m/v in sea water) 4% peptone, 1.4% KH_2PO_4, 0.24% K_2HPO_4, 0.04% $MgSO_4{\cdot}7H_2O$, 0.4% NaCl, 2% Olive oil at pH 8.0. The 50 ml fermentation medium was inoculated with 6 mm diameter disc obtained from 7 days cultured Petri dishes containing spores of the strains (SW1 to SW6) (Colla et al., 2016). The fermentation mediums were incubated at 30 °C for 8 days by shaking at 120 rpm. The samples were collected at 24 h intervals and centrifuged at 10,000× *g* for 10 min. The culture filtrate was considered as crude extracellular lipase and was used for lipase assays.

2.5. Estimation of the total protein content

Total protein content in the culture filtrates was estimated according to Bradford (1976) by using Bovine serum albumin (BSA) to generate the standard curves.

2.6. Qualitative screening of lipase

2.6.1. Analysis of the hydrolytic activity of lipolytic fungi

The hydrolytic activity of culture filtrate was assayed as previously described by Krieger et al. (1999) using para nitrophenyl palmitate (*p*-NPP) as substrate. Briefly, 20 µL of culture filtrate was added to 380 µL of the substrate solution consisting of one part of solution A (3.0 mM *p*-NPP in 2-propanol) and nine parts of solution B (100 mM Tri-HCl buffer, pH 8, 0.4% Triton X-100% and 0.1% gum Arabic), which was freshly prepared before use. The reaction was stopped by boiling for 10 min, followed by centrifugation at 8000 × *g* for 10 min. The release of para nitrophenol (*p*-PN) was measured using EnSpire Multimode Plate Reader at 410 nm against a blank containing only buffer. One unit of enzyme activity is defined as the amount of enzyme that released 1 µmole *p*-PN per minute. Calculation of lipase activity in units was done by using the standard curve of *p*-PN.

2.6.2. Analysis of the esterification activity of fungi

For identifying fungi displaying esterification activity, we employed a method according to Wu et al. (1996b) with some modifications. Briefly, the reaction mixture contains 0.77 mM ethanol and 0.70 mM oleic acid as substrates, 1 ml of lyophilized filtrates from selected fungal strains was taken as biocatalyst and kept shaking at 200 rpm and 37 °C. Samples withdrawn at 3 h intervals were analyzed by titration procedure as previously reported (Marchetti and Errazu, 2008). To measure the residual activity (free acid), the withdrawn amount of sample was dissolved in 1:1 ethanol and diethyl ether (v/v), a few drops of 0.1% phenolphthalein was added as indicator and titration was performed using 0.02 M KOH. The amount of KOH consumed was recorded and acidity was calculated using the following equation:

$$A = (V\ 1000\ Mw\ C)/W$$

Where A: acidity index; V: volume of KOH solution employed for titration, ml; Mw: molecular weight of the KOH, g/mol; C: concentration of the KOH solution used for titration, mol/l; W: weight of the sample taken for analysis, mg.

The conversion of free fatty acid was calculated using the following.

$$X_{ffa} = (a_i - a_t)/a_i$$

Where a_i is the initial acidity of the mixture and a_t is the acidity at time 't'.

2.6.3. Analysis of the transesterification activity of fungi

Identification of fungi displaying transesterification activity was performed according to the previously published method (Teng and Xu, 2007; Escobar-Niño et al., 2014). Briefly, 1.0 ml of culture filtrates were withdrawn after every 24 h and lyophilized for 24 h. The lyophilized culture filtrate was mixed with 1 ml of 10 mM *p*-NPP (in n-hexane) and 60 µL of anhydrous ethanol. The mixture was tightly sealed and suspended in a rotary shaker at 120 rpm and 37 °C for 12 h. A mixture of anhydrous ethanol and *p*-PNP without lyophilized culture filtrate was taken as negative control whereas a mixture of *p*-PNP with lyophilized