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Isolation, Identification and Optimization of a New Extracellular Lipase Producing Strain of *Rhizopus* sp.

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Abstract A lipolytic mesophilic fungus which produces lipase extracellularly was isolated from soil. Based on ITS1-5.8S–ITS4 region sequences of ribosomal RNA, it was concluded that the isolate JK-1 belongs to genus *Rhizopus* and clades with *Rhizopus oryzae*. The present paper reports the screening, isolation, identification, and optimization of fermentation conditions for the production of lipase (EC 3.1.1.3). Culture conditions were optimized, and the highest lipase production was observed in basal medium with corn steep liquor as nitrogen source and glucose as carbon source. Maximum lipase production was observed at 72 h, which is about 870 U/ml. Optimization of fermentation conditions resulted in 16-fold enhancement in enzyme production.

Keywords *Rhizopus* · ITS region · Lipase · Medium optimization · Fermentation · Corn steep liquor

Introduction

Lipases (glycerol ester hydrolyses EC 3.1.1.3) are having wide importance in industry. Lipases catalyze the hydrolysis of triglycerols into free fatty acids, glycerol as well as mono and di-acyl glycerols. Another interesting feature of lipase is its ability to synthesize ester bonds in nonaqueous medium [2, 10–12] which facilitates transestrification of triglycerides through the process of transestrification [26]. Transestrification of fats and oils by lipases gives alkyl esters of fatty acids which can be used as biodiesel [10, 16]. Along with this emerging role, lipases have various commercial applications in different industries such as food, fine chemicals, detergents, waste water treatment, cosmetics, pharmaceuticals as well as leather processing [2, 12].

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There are various microbial sources of lipase producers among which fungi are preferred when used in industrial applications. Further purification of enzyme becomes easier if the enzyme is produced extracellularly [22]. Lipase activity has been detected in various species of *Rhizopus* such as *Rhizopus oryzae* [9, 11, 25], *Rhizopus homothallicus* [5], *Rhizopus oligosporus* [13, 21], *Rhizopus delemar* [14], and *Rhizopus japonicas* [3]. *Rhizopus* is well reported for the production of lipase; however, there are very few reports on optimization of fermentation conditions for lipase production [5, 8, 11, 18].

In the present investigation, we have screened, isolated, and identified a novel mesophilic fungus *Rhizopus* strain JK-1 from soil samples of oil spillage areas which produces extracellular lipase constitutively. The culture conditions were optimized for growth and maximum lipase production. The production of high titer of any enzyme by optimizing the growth parameters is of prime importance in enzymology. In this manuscript, we report mesophilic *Rhizopus* sp. producing highest extracellular lipase units under optimum conditions.

Materials and Methods

Source and Isolation of Lipase Producer

Five different soil samples were collected from places contaminated with oil near lamps in the local temples from Pune, India. The samples were collected in 10 ml sterile MGYP containing *Pongamia* oil (3%). The tubes were incubated at 30 °C on a rotary shaker at 180 rpm for 24 h. Further isolation was done on PDA plates.

Rapid Screening of Lipase Producer

Fifteen different fungal isolates were screened for lipase production on Rhodamine B agar plates. The fungal isolates were inoculated on media of the following composition (g/L) Nutrient broth (Hi Media, India), 8.0; sodium chloride, 4.0; agar, 10.0. The medium was adjusted to pH 7.0, autoclaved and cooled to 60 °C. Olive oil (31.25 ml) and 10 ml of Rhodamine B solution (0.001%, w/v) were added with vigorous stirring and emulsified by mixing for 1 min. Plates were incubated at 30 °C for 48 h and irradiated with UV light at 350 nm for orange fluorescent halo which indicates lipolytic activity of the isolate [17]. Isolate which showed significant zone of fluorescence indicative of lipase production, was selected for further studies.

Lipase Assay

The activities of enzyme was analyzed spectrophotometrically measuring the increment in the absorption at 410 nm promoted by the hydrolysis of pNPP [28]. The reaction mixture consisted of 0.1 ml of diluted enzyme sample, 0.9 ml of pNPP substrate solution and 1 ml of phosphate buffer (0.05 M, pH 7.0). It was incubated at 30 °C for 30 min, followed by addition of 2 ml 2-propanol. The absorbance was measured at 410 nm. The substrate solution containing 30 mg pNPP, 10 ml 2-propanol, 0.1 ml Triton X-100 in 100 ml phosphate buffer (0.05 M, pH 7.0) was prepared freshly. The lipase unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPP per minute under the described conditions. The protein in supernatant was estimated by Folin–Lowry method [20]. Bovine serum albumin (BSA) was used as standard.

Lipase Production

Lipase production by strain JK-1 was conducted in 250 ml Erlenmeyer flasks with 50 ml of the basal medium containing Glucose 1%, Na_2NO_3 0.1%, $MgSO_4$ 0.05%, KH_2PO_4 0.1%, Peptone 3% inoculated with 10% seed culture prepared in malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, and glucose 2%. Flasks were incubated at 30 °C on rotary shaker with 180 rpm for 6 days. Enzyme production was monitored after every 24 h. Extracellular enzyme from the flask was harvested by centrifugation at 5,000 rpm for 15 min and supernatant was used as extracellular enzyme source.

Biomass

Mycelial dry weight was determined by filtering the culture medium, dried to a constant weight at 80 °C. The difference in weight denoted the mycelial growth of fungus.

Identification

DNA Isolation

DNA was isolated by using the method by Sambrook and Russel [15].

DNA Amplification and Sequencing

Polymerase chain reaction (PCR) amplification conditions using primers for internal transcribed spacer (ITS) region were 35 PCR cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 1 min [4]. Amplified DNA was checked on 1% agarose gel and purified using the PEG-NaCl method. The purified PCR product was rechecked on 1% agarose gel and used for sequencing using ABI Bigdye chemistry version 3.1. Sequences acquired were quality checked manually. CromasPro version 1.34 was used for contig formation and quality trimming.

Nucleotide Sequence Submission and Phylogenetic Analysis

The contig obtained was deposited in NCBI Genbank database available under the accession no HQ222811. Sequences homologous to JK-1 were obtained using NCBI BLAST. Sequences with high query coverage and homology were selected for phylogenetic analysis [23]. Multiple sequence alignment was done using ClustalX [19], the aligned sequences were trimmed using DAMBE and the phylogenetic tree was constructed using MEGA version 4 [27].

Optimization of Fermentation Parameters

Optimization of different nutrient and physical parameters for lipase production were studied by maintaining all factors constant except the one being studied. Fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of sterilized basal media. Effect of inoculum size on lipase production was studied by inoculating the basal medium with 5–20% inoculum. The inoculum size which gave maximum lipase production was used for further studies. Flasks were kept on shaker at 180 rpm, 30 °C for 120 h.

Effect of pH on lipase production was studied by cultivating the isolate in different initial pH values of the basal medium. Effect of temperature on enzyme production was studied by incubating the organism at various temperatures ranging from 25 to 45 °C. Effect of aeration was studied by incubating the fungus in the 250 ml Erlenmeyer flasks containing basal medium ranging from 25 to 125 ml.

The effects of different medium components such as carbon and nitrogen sources were studied. Different carbon sources (1%) studied for growth and lipase production were glucose, sucrose, maltose, glycerol and olive oil. The influence of different nitrogen sources (3 g%) such as peptone, tryptone, casamino acids, yeast extract and corn steep liquor (CSL) was studied by supplementing in basal medium along with the optimized carbon source. Basal medium without carbon and nitrogen source was considered as control and all the experiments were run in triplicates.

Results

Source, Screening and Isolation of Lipase Producer

Five samples were collected from different oil contaminated soil near lamps in the local temples from Pune, India and enriched in malt extract, glucose, yeast extract and peptone (MGYP) with pongamia oil. Among these samples, 15 fungal isolates were screened for lipase production on Rhodamine B agar plates. The best lipase producer with significant fluorescence halo was selected for maximum lipase production in basal medium at 30 °C for 6 days.

Identification of Lipase Producer

The phylogenetic analysis of strain JK-1's ITS sequences, a complete stretch of 553 base pairs, revealed that strain JK-1 clades with *R. oryzae* (Fig. 1). The sequence of JK-1's ITS domain is available in NCBI database under the GenBank (HQ222811).

Optimization of Fermentation Parameters

This study was done to increase the yield of lipase production from *Rhizopus* strain JK-1. Optimum lipase production was observed in basal medium containing glucose 1%, Na_2NO_3 0.1%, $MgSO_4$ 0.05%, KH_2PO_4 0.1%, and peptone 3%. This medium was used for the rest

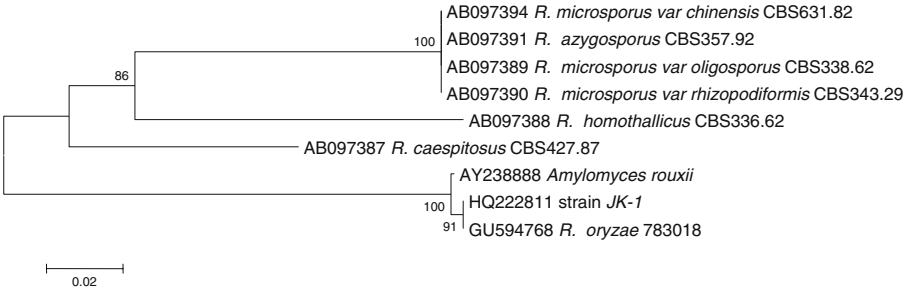

Fig. 1 Neighbor-joining phylogenetic tree constructed based on ITS sequences, showing the phylogenetic relationship between Strain JK-1 and other *Rhizopus* sp. Bootstrap values (expressed as percentages of 1,000 replications)>50% are given at nodes. The *bar* represents two substitutions per 100 nucleotides

of the experiments. Ten percent inoculum gave maximum lipase production (data not shown; 750 U/ml). As shown in Fig. 2, the production of lipase increased steadily with the cultivation time and the best enzyme production, about 802 U/ml was reached at 7.1 g/l mycelial dry weight after 3 days of cultivation. After 3 days, gradual decrease in enzyme activity was seen. Increase in biomass was seen up to 4 days, which enters stationary phase and simultaneously enzyme activity goes decreasing. The pH profile for lipase from *Rhizopus* strain JK-1 was determined in basal medium adjusted to different pH values ranging from 5.5 to 8.5. Optimum lipase activity was detected at pH 7.5 (Fig. 3). Temperature profile for lipase activity showed that enzyme activity was maximum at 30 °C when incubated at different temperatures ranging from 25 to 45 °C. The culture failed to grow at 25 and at 45 °C (Fig. 4). Effect of aeration was studied by dispensing 25–125 ml of basal medium in 250 ml Erlenmeyer flasks. Maximum lipase production was observed in the flasks containing 50 ml basal medium when incubated at 30 °C, pH 7.5, at 180 rpm (Fig. 5).

Medium composition was optimized to maximize the lipase production. Glucose was observed as best carbon source among the tested simple and complex carbon sources (Fig. 6). However, there was increased biomass production in olive oil containing basal medium (data not showed), but no considerable increase in enzyme activity was observed. Presence of favorable nitrogen source has its own significance on lipase production, which was studied using different nitrogen sources such as peptone, tryptone, casamino acids, yeast extract, and CSL. CSL (3 g%) was found to be the best among the nitrogen source tested which gave the best enzyme units up to 870 U/ml (Figs. 7 and 8) with specific activity in the range of 28–31 U/mg throughout the study. Presence of glucose and CSL increased lipase activity up to 16-folds as compared with control which is a same basal medium without carbon and nitrogen source.

Discussion

Strain JK-1 was assigned to genus *Rhizopus* based on the phylogenetic analysis of ITS1-5.8S-ITS 4 region sequence, a complete stretch of 553 base pairs [1]. The sequence of JK-1's ITS domain is available in NCBI database. The property of *Rhizopus* strain JK-1 to produce extracellular lipase is having its own significance over reported intracellular lipases [6] by minimizing the downstream processing of enzyme. There are reports on lipase

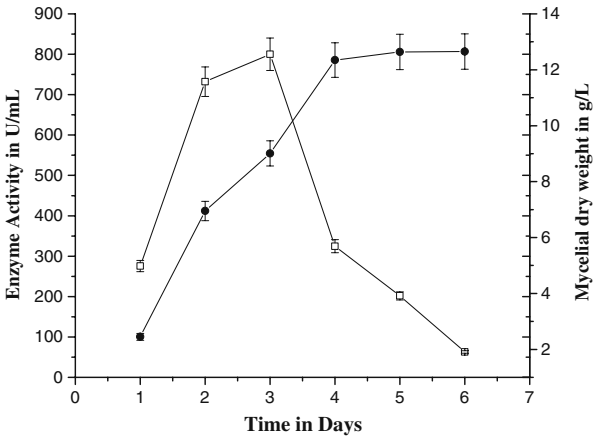

Fig. 2 Effect of fermentation time on lipase and biomass production at 30 °C, pH 7.5, 180 rpm in basal medium with CSL. Samples were removed at different intervals and assayed for growth (●) and lipase activity (□) simultaneously

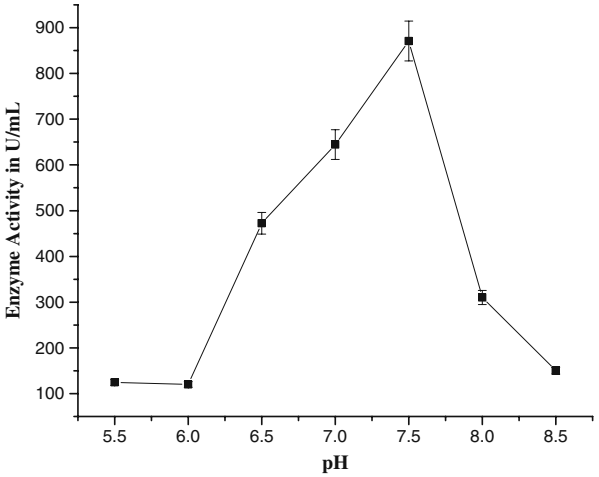

Fig. 3 Effect of initial pH of the culture medium on lipase production by strain JK-1

production by *Rhizopus* sp. but the enzyme unit activity reported is very low under optimum fermentation conditions [5, 9, 11, 13, 25]. Our isolate *Rhizopus* strain JK-1 produces this enzyme extracellularly with 862 U/ml (specific activity 30.38 U/mg) under optimum conditions. As per our knowledge, this is second highest lipase activity report when compared with species so far reported [3, 5, 6, 9, 11, 13, 14, 21, 25]. Microbial lipase fermentations are affected by the medium pH, temperature, media composition, inoculation volume, and aeration. Our isolate *Rhizopus* strain JK-1 can grow in the temperature range of 30–45 °C and pH 5.5–9.0. Although the culture grows at higher temperatures, maximum enzyme production was obtained at 30 °C. This is the second highest activity of lipase enzyme produced (extracellular) by *Rhizopus* sp. The previous report is by Essamri et al. [6] (activity 3,032 IU/g), although it is produced intracellularly.

The production of enzyme with very high activity is possible only after optimizing the growth parameters which is a typical enzymological exercise. The optimization of various nutritional and physical parameters to which an organism is exposed is known to

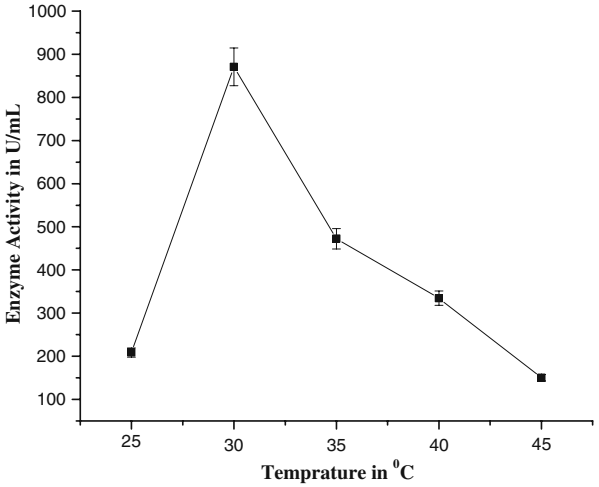

Fig. 4 Effect of incubation tem perature on lipase production by strain JK-1

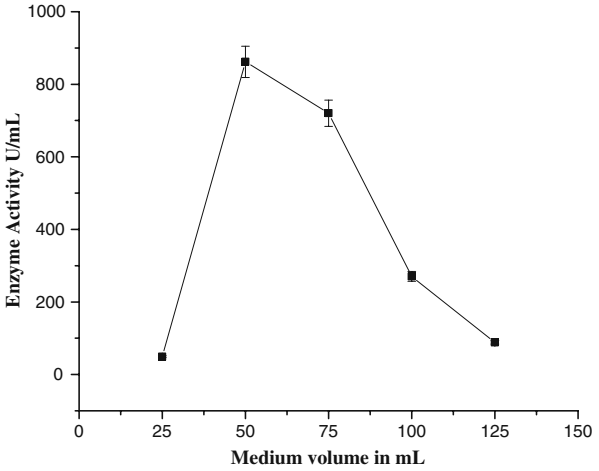

Fig. 5 Effect of aeration on lipase production by strain JK-1

significantly increase the product yield. Various nutritional and physical parameters are known to be involved in increasing the enzyme activity, and it is already known that the culture environment has a dramatic influence on enzyme production [7]. Yield of the enzyme can be enhanced several folds by providing the suitable cultural conditions. Since lipase is an industrially important enzyme, its production needs to be increased by optimizing the various parameters. A 16-fold increase in lipase production was achieved in basal medium with CSL (3 g%), pH 7.5, incubated at 30 °C at 180 rpm for 72 h.

Initial pH is a very important factor which influences the extracellular enzyme production. In case of fungi, majority of researchers have reported an acidic pH to be the most appropriate for enzyme production [11]. However, Diaz et al. [5] and Nahas [21] reported that pH 6.5 is optimum for lipase production by *R. homothallicus* and *R. origosporus*. We are reporting slightly near and above neutral, pH (pH 7.5), for optimum lipase production. The effect of aeration on fungi was important for optimum metabolic rates with respect to growth and enzyme production. For aerobic fermentation, oxygen transfer is a key variable and is a function of aeration and agitation. Medium volume plays

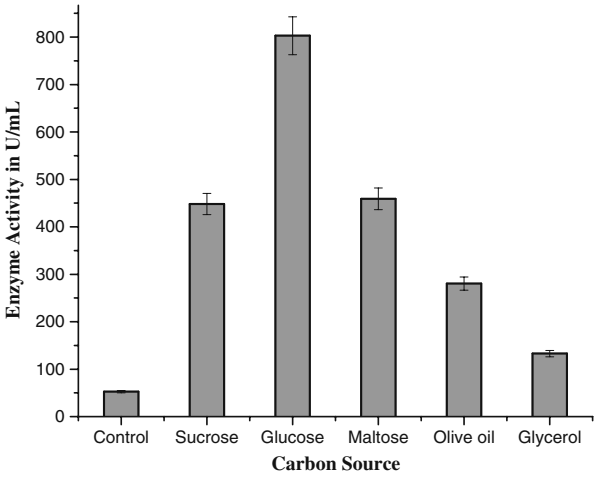

Fig. 6 Effect of carbon source on lipase production during cultivation of strain JK-1

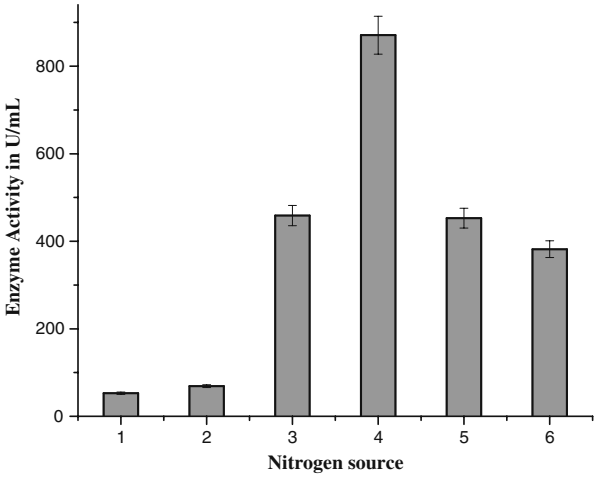

Fig. 7 Effect of nitrogen source on lipase production during cultivation of strain JK-1. The following different nitrogen sources were used: *1* control, *2* cassamino acids, *3* peptone, *4* csl, *5* tryptone, and *6* yeast extract

an important role in the mass transfer during fermentation. Low oxygen supplies can alter fungal metabolism and, consequently, the production of lipases [7]. These facts were applied for lipase production of *Rhizopus* strain JK-1. We obtained maximum enzyme production (862 U/ml, with specific activity of 30.38 U/mg) when 50 ml of basal medium was dispensed in 250 ml Erlenmeyer flasks.

In industrial scale fermentation, use of inducers may cause inconsistencies in medium homogeneity and oil–water surface problems, and this may involve expensive purification process [24]. However, *Rhizopus* strain JK-1 produces lipase constitutively. One percent glucose in the basal medium showed maximum lipase production. Therefore, an efficient microbial system that can utilize soluble sugars as carbon and energy sources is quite attractive, since the enzyme production is constitutive. Among the various nitrogen sources tested, CSL, at concentration of 3 g%, was the best for optimum enzyme production. The nitrogen source is usually the most expensive component of the medium, when organic

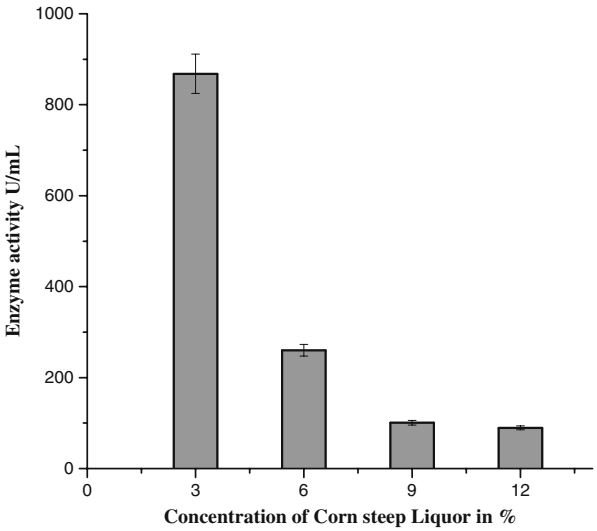

Fig. 8 Effect of CSL concentration on enzyme production

nitrogen sources such as peptone, tryptone, yeast extract, etc., are considered. Various *Rhizopus* sp. have been reported using CSL as nitrogen source but in very high concentration up to 6–7 g% [6]. Cost of lipase production can be reduced considerably by the use of inexpensive raw materials, such as CSL (at 3 g% concentration), prompting new industrial applications.

In the conclusion, we report a mesophilic *Rhizopus* JK-1 which constitutively produces extracellular lipase. Maximum lipase production was seen when basal medium was supplemented with 1% glucose and 3% CSL with optimum pH, temperature, aeration, and incubation time. We are reporting highest lipase units in these optimum conditions, prompting cost-effective industrial production of this enzyme.

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