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Investigation of Lipase Production by Milk Isolate *Serratia rubidaea*

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Summary

Production of extracellular lipase in submerged culture of *Serratia rubidaea* has been investigated. The lipase production was optimized in shake flask experiments. The observed pH and temperature range optimum for maximum lipase production were 7–8 and 30–40 °C, respectively. With a selected nitrogen source, casein ((6.5±0.015) U/mL) and soytone ((9.4±0.02) U/mL) were suitable substrates for accelerating lipase production. The optimized concentration of casein and soytone was 24 g/L ((9.95±0.02) U/mL) and 5 g/L ((14.8±0.03) U/mL), respectively. The effect of carbon source on lipase production indicated that starch was suitable substrate to maximize lipase production ((15.60±0.20) U/mL) and the optimum concentration registered was 4 g/L ((17.46±0.20) U/mL). Investigating the effect of lipids and surfactants showed that the gingily oil ((20.52±0.20) U/mL) and Tween 20 ((27.10±0.01) U/mL) were suitable substrates for maximizing lipase production, and the optimum concentrations registered were 15 mL/L ((23.15±0.24) U/mL) and 6 mL/L ((34.20±0.01) U/mL), respectively. Partial purification of lipase indicated that the molecular mass of partially purified enzyme was 54 kDa.

Key words: lipase, casein, soytone, gingily oil, Tween 20, *Serratia rubidaea*

Introduction

Lipases or triacylglycerol acyl ester hydrolases are carboxylesterases that catalyze both hydrolysis and synthesis of esters formed from glycerol. Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential (*1*). Lipases find application in many areas of biotechnology due to their ability to catalyse enantioselective reactions with a wide range of substrates and their stability over wide variations of temperature and pH (*2*). Lipases have been found in many species of animals, plants and microorganisms. A wide range of microorganisms (bacteria, fungi and yeast) can produce lipases with different enzymological properties and substrate specificities (*3*). In particular, lipases from fungi are important in industrial applications (*4*). A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal lipases. Amongst the lipase-producing organisms, *Bacillus, Candida, Penicillium, Pseudomonas, Rhizomucor* and *Rhizopus* spp. are the outstanding ones (*3*).

Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and are non-specific in their substrate specificity and a few bacterial lipases are thermostable (*5*). Among bacteria, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Pseudomonas*, *Staphylococcus* and *Chromobacterium* spp. have been exploited for the production of lipases. But in recent years, *Serratia* sp. has been studied for its ability to produce lipase (*6*).

Microbial lipases form a versatile tool in biotechnology and in recent years they have become an important

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class of enzymes. Their applications include the addition to detergents, manufacture of food ingredients, pitch control in pulp and paper industry (*7*), production of aromas, production of insecticides, synthesis of drugs such as naxopren and ibuprofen, and as a biocatalyst of stereoselective transformations (*2*). With this upsurge, the exponential increase in the application of lipases in various fields in the past few years demands both qualitative and quantitative improvement. The quantitative enhancement requires strain improvement and medium optimization for overproduction. The spectacularly successful examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection.

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acids, fatty acid alcohol or fatty acid ester for induction. Triacyl glycerols are the main substrates of lipases, they are uncharged lipids. The triacyl glycerols with long-chain fatty acids esterified with glycerol are insoluble in water, although those with short-chain fatty acids are sparingly soluble in water (*8*). Lipase biosynthesis by microorganisms can be enhanced by optimization of culture condition factors such as temperature, pH, aeration, medium composition, *etc*. and sometimes these conditions may affect extracellular lipase production. The presence of some compounds, *i.e.* fatty acids, triglycerides or surfactants has often been seen to induce lipase secretion (*8*). Therefore, the current study was undertaken to optimize the lipase production by locally isolated *Serratia rubidaea* on relatively low cost media.

Materials and Methods

Microorganism and lipolytic activity

The bacterial strain was isolated from raw milk sample and identified as *Serratia rubidaea* by standard procedure described in Bergey's Manual of Determinative Bacteriology (*9*). The identified organism was tested for lipase production on Spirit Blue agar plates supplemented with tributyrin. After 4 days of incubation at 32 °C, the lipolytic activity was confirmed by forming a clear zone around the colony. The lipolytic activity in the liquid medium was assessed first by enriching *S. rubidaea* in the enrichment medium containing beef extract 0.3 %, peptone 0.5 %, NaCl 0.5 % and glucose 0.5 % at pH=7 and then 10 % of enriched culture was inoculated in 250-mL flask containing 45 mL of basal TS broth (tryptone 16 g/L, soytone 3 g/L, NaCl 5 g/L, glucose 2.5 g/L, K_2HPO_4 2.5 g/L) (*10*). The culture was then incubated for 4 days by reciprocal shaking at 32 ºC. The cells were then harvested by centrifugation at 10 000 rpm for 15 min and the supernatant was used for further assay. The same culture conditions were followed for all the optimization experiments, and were carried out in triplicate. During the optimization, the experiments were performed in a consecutive manner by incorporating previously optimized parameters. The last optimization experiment was also performed under the previously optimized conditions. The interrelating effect of all optimized factors in this experiment was assessed.

Lipase assay

The lipase activity in the culture supernatant was determined by titrimetry (olive oil emulsion method) (*11*). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of equivalent fatty acid/(mL·min) under the standard assay conditions.

Effect of pH and temperature on lipase production

The effect of pH and temperature on lipase production was determined by incubating the culture flasks with different pH (4, 5, 6, 7, 8, and 9) and temperature (10, 20, 30, 40, 50, 60, 70, and 80 °C).

Optimization of medium components for lipase production

The optimization of medium components with suitable nutrient sources was carried out at the optimum pH and temperature, by substituting components present in the basal medium and subsequent optimization.

Effect of nitrogen sources on lipase production

To test the effect of nitrogen sources on lipase production, six different nitrogen sources such as yeast extract, skim milk powder, casein, protease peptone, beef extract and urea were used. They were individually tested by replacing the tryptone present in the basal medium at the concentration of 16 g/L. After screening, the maximum lipase-yielding substrate was then taken for optimization at various concentrations (16, 18, 20, 22, 24, 26, 28, 30, 32, 34 and 36 g/L). To test the effect of additional nitrogen source on lipase production, six different nitrogen sources were screened: yeast extract, skim milk powder, beef extract, protease peptone, tryptone and urea. They were tested individually instead of the soytone, which was present in the basal medium at the concentration of 3 g/L. After screening, the maximum lipase-yielding substrate was taken for further optimization by varying its concentration (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 g/L).

Effect of carbon sources on lipase production

The effect of carbon sources on lipase production was tested by using five different carbon sources, namely sucrose, fructose, lactose, galactose and starch. They were tested individually by replacing the glucose present in the basal medium at the concentration of 2.5 g/L. Then the maximum enzyme-producing carbon source was further optimized by varying its concentration (2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 g/L).

Effect of triglycerides on lipase production

The lipase production was accelerated by incorporation of different lipid sources, namely olive oil, coconut oil, gingily oil, tributyrin, and cod liver oil, in the optimized medium. They were tested individually at the fraction of 5 mL/L and the medium without oil was used as the control. The triglyceride producing maximum lipase was then optimized by varying its fraction (5, 10, 15, 20 and 25 mL/L).

Effect of surfactants on lipase production

The surfactant that induces the production of lipase was detected by incorporating different surfactants, namely Tween 20, Tween 80, polyethylene glycol 300, Triton X 100 and Criton X 100 in the optimized medium. They were tested individually at the fraction of 2 mL/L and the medium without surfactant was used as control. The surfactant producing maximum lipase was then optimized by varying its fraction (2, 3, 4, 5, 6, 7, 8, 9 and 10 mL/L).

Partial purification of lipase

The test strain was first enriched on the enrichment medium containing beef extract 0.3 %, peptone 0.5 %, NaCl 0.5 % and glucose 0.5 % at pH=7 and then cultured in nutrient-optimized experimental medium at room temperature for 4 days by reciprocal shaking. Then, after 4 days of growth, the culture filtrate was collected separately by centrifugation. A volume of 100 mL of crude enzyme was taken individually, and 500 mL of ethyl alcohol were added. The mixture was allowed to precipitate for an hour and then centrifuged at 5000 rpm for 15 min. The precipitate was collected by centrifugation at 1000 rpm for 15 min. After that the precipitate was dissolved in minimum amount of 20 mL Tris/HCl buffer (pH=8), containing $CaCl_2$ and dialyzed against the same buffer for 24 h. The dialyzate was collected in a sterile container and stored at 4 °C for further analysis. For the determination of molecular mass of partially purified lipase, 15 % SDS-PAGE was performed. The R_f values of the fractions and percentage of raw volume were calculated by using molecular mass markers, and gel documentation system (Syngene, UK).

Statistical analysis

The results obtained in the present study were subjected to relevant statistical analysis (*12*).

Results and Discussion

The bacterium *S. rubidaea* isolated from raw milk showed lipolytic activity after 2 days of incubation at 32 °C on Spirit Blue agar plates. The diameter of the zone was 15 mm.

Effect of pH on lipase production

The effect of the pH of the medium on lipase production by *S. rubidaea* indicated a linear increase from (1.18±0.012) to (4.0±0.01) U/mL corresponding to the increase of pH from 4 to 8. At the pH of 9 and 10, the lipase production decreased ((3.83±0.07) and (2.71±0.03) U/mL), respectively. Invariably, the lipase production by *Serratia marcescens* prefers slightly acidic pH (6.5–7) (*13*) and *Pseudomonas aeruginosa* MB prefers neutral pH (*14*). The present study revealed that the lipase production by *S. rubidaea* requires alkaline pH. Lipase activity by *Serratia grimesii* was high in pH=8–9 (*15*). The lipase activity by *S. marcescens* was found to be maximum at pH=8 (*6,16*). One-way ANOVA test conducted to obtain the data on lipase production as a function of medium pH revealed a highly significant variation ($p<0.0001$).

Effect of temperature on lipase production

Experiment on the effect of temperature indicated that the lipase production of *S. rubidaea* was maximum ((4.29±0.001) U/mL) at the optimum temperature of 40 °C. But below (10 to 30 °C) and above (50 to 80 °C) the optimum temperature, the lipase production recorded was low ((1.9±0.02) to (3.59±0.033) and (2.10±0.05) to (3.91±0.06) U/mL, respectively). Lipase production by *S. marcescens* was higher at the cultivation temperature of 25 °C, compared to 30 and 35 °C (*13*). Similarly, the lipase production observed in *Pseudomonas aeruginosa* MB was higher at 30 °C. Earlier studies regarding lipase production by *Serratia* sp. isolated from raw milk samples stated that optimum temperature for lipase production was 30–40 °C (*15*), but in the present study lipase activity showed gradual increase with the increase of temperature from 10 to 40 °C and further increase of temperature, beyond 40 °C, decreased the production of lipase. Statistical analysis by one-way ANOVA test for the data on lipase activity as a function of medium temperature changes indicated a highly significant variation ($p<0.0001$).

Effect of nitrogen sources on lipase production

In the present study different nitrogen sources (either organic or inorganic) were screened at a fixed concentration of 16 g/L. The lipase production was greatly influenced by the tested nitrogen sources (Fig. 1). Among these nitrogen sources, casein produced maximum lipase ((6.5±0.015) U/mL), compared to others. This is because casein is a simple milk protein and can be easily utilized by a candidate species. After casein, higher lipase production was registered in tryptone-supplied basal medium, followed by others. The lowest lipase production ((2.93±0.05) U/mL) was registered in yeast extract-supplied medium. A possible mechanism may be that yeast extract is a complex nitrogen source and thus requires the cells to secrete more protease for its enzymatic degradation before utilization. This might result in lower production and higher degradation of the extracellular lipase (*13*). One-way ANOVA revealed that the lipase production at varying nitrogen sources was found to be statistically significant ($p<0.0001$).

Lipase production by *S. rubidaea* was also influenced by the concentration of casein in the medium. In the medium with 18 to 36 g/L of casein (added at the interval

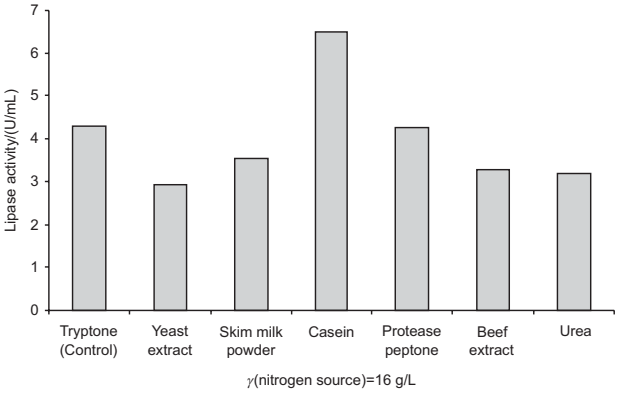

Fig. 1. Screening of nitrogen sources for lipase production

of 2 g), maximum lipase production ((9.95±0.015) U/mL) was registered at the concentration of 24 g/L. But at low (18 to 22 g/L) and high (26 to 36 g/L) casein concentrations, the lipase production was lower ((6.98±0.06) to (8.70±0.031) U/mL and (7.76±0.02) to (5.42±0.011) U/mL, respectively). The statistical analysis by one-way ANOVA test revealed that the lipase production at various concentrations of casein was found to be statistically significant ($p<0.0001$).

Effect of additional nitrogen source on the production of lipase by *S. rubidaea* revealed that the basal medium supplied with soytone gave its maximum production ((9.40±0.02) U/mL). In consistence with the present study, Kim *et al.* (*10*) reported that the lipase production by *Pseudomonas* sp. S34 was maximum with two nitrogen sources, namely trypton and soytone, and the studies by Lima *et al.* (2) reported that lipase production by *Penicillium aurantiogriseum* was high when using inorganic nitrogen source, but a medium with two organic nitrogen sources displayed lipase production more or less the same to that of the medium containing one organic nitrogen source. In the present study, in addition to soytone, medium supplied with skim milk or tryptone also gave more or less similar production results (Fig. 2). The statistical analysis by one-way ANOVA showed that the influence of nitrogen sources on total lipase production was statistically significant ($p<0.0001$).

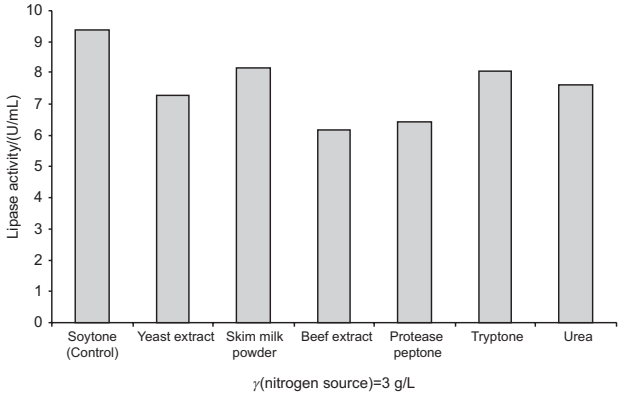

Fig. 2. Screening of additional nitrogen sources for lipase production in combination with casein

The effect of different concentrations of soytone on lipase production showed a positive linear increase ((9.9±0.034) to (14.8±0.03) U/mL) with respect to the increase in the concentration of soytone from 3.5 to 5.0 g/L at 0.5 g interval. Further increase in the concentration of soytone to 6.5 g/L resulted in decreased lipase production ((12.2±0.011) U/mL). One-way ANOVA indicated that the influence of various concentrations of soytone on the lipase production was found to be statistically significant ($p<0.001$).

Effect of carbon sources on lipase production

A range of different carbon sources, mainly carbohydrates, were screened for their efficiency to support lipase production by *S. rubidaea* at the fixed concentration of 2.5 g/L. On the basis of lipase activity, it was concluded that the medium containing starch was more suitable for maximum lipase ((15.60±0.20) U/mL) production than other carbon sources (Fig. 3). The influence of different carbon sources on the production of lipase was found to be statistically significant ($p<0.0001$) by the one-way ANOVA. This result supports the earlier observations of lipase production by *Pseudomonas fragi* 2239B (*17*) stimulated with polysaccharides and higher lipase production by *Bacillus circulans* induced with starch (*18*). The lipase production by *Streptomyces* sp. and *P. fluorescens* was increased when these organisms were cultured in the medium containing soluble starch (*18*).

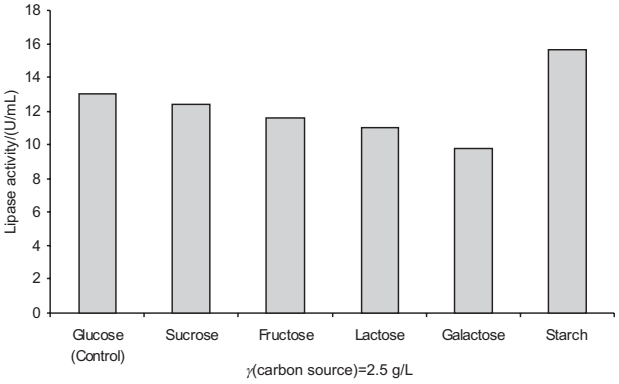

Fig. 3. Screening of carbon sources for lipase production

In the medium with added starch *S. rubidaea* lipase production depended on its concentration. Among the tested starch concentrations in the range from 3.0 to 6.5 g/L, at the interval of 0.5 g/L, the lipase production was maximum ((17.46±0.20) U/mL) at 4.0 g/L. When low (3.0 to 3.5 g/L) or high (4.5 to 6.5 g/L) concentrations of starch were added, the lipase production was lower ((16.2±0.23) to (16.76±0.27) U/mL and (16.5±0.11) to (14.6±0.02) U/mL, respectively). One-way ANOVA test revealed that the influence of various concentrations of starch on lipase production was found to be statistically significant ($p<0.0001$).

Influence of lipid sources on lipase production

Lipid-induced lipase production by *S. rubidaea* was confirmed by the addition of lipids to the culture medium. Here, the lipase production in lipid-supplemented medium gave better production than the control medium. On the basis of the activity, the gingily oil was found to be suitable for maximizing the lipase production ((20.52±0.20) U/mL) by *S. rubidaea* (Fig. 4). This is because triglycerides are important substrates for lipase production as they can act as an inducer as well as an inhibitor. In the present study, all the tested triglycerides were found to induce the lipase synthesis with different level of enzyme production. This study is in agreement with the previous work on castor oil-induced lipase production by *Pseudomonas aeruginosa* KKA-5 (*19*), sunflower oil and olive oil-induced extracellular lipase production by *Yarrowia lipolytica* (*8*) and vegetable oil-induced lipase production by *Candida rugosa* (DSM 2031) (*20*). One-way ANOVA showed that the influence of various lipid

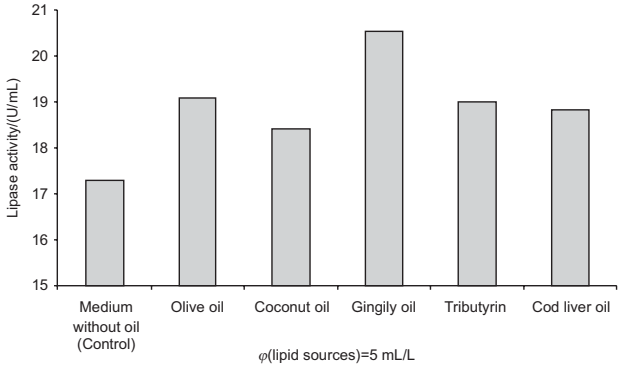

Fig. 4. Influence of various triglycerides on lipase production

sources on total lipase production was statistically significant ($p<0.05$). Addition of gingily oil in the fraction range of 5 to 25 mL/L at an interval of 5 mL/L indicated that the lipase production was optimum ((23.15± 0.24) U/mL) in the medium containing 15 mL/L of gingily oil. At low (5 mL/L) and high (25 mL/L) fractions of gingily oil, the lipase production was reduced ((18± 0.05) and (19.98±0.15) U/mL). One-way ANOVA results of lipase production at different fractions of gingily oil were found to be statistically significant ($p<0.0001$).

Effect of surfactants on lipase production

The studies on the influence of surfactants on lipase production revealed that maximum lipase production by *S. rubidaea* was induced by Tween 20 ((27.10±0.01) U/mL), followed by polyethylene glycol 300 ((26.00±0.06) U/mL). Thus, all the tested surfactants showed positive influence on lipase production compared to the control. Similarly, the studies of lipase production by *P. aeruginosa* EF2 indicated the positive influence of Tween 80 (*21*). The production of lipase by *Rhizophus oligosporus* induced by Tween 20 was reported by Johri *et al.* (*22*), while surfactant-induced lipase production by *Yarrowia lipolytica* was studied by Dominguez *et al.* (*8*). From the present study it is also evident that the lipase production by microorganisms could be induced by the addition of surfactants (Fig. 5). One-way ANOVA test for lipase production showed that the use of different surfactants was found to be statistically significant ($p<0001$). Addition of surfactants to the medium also showed that the lipase

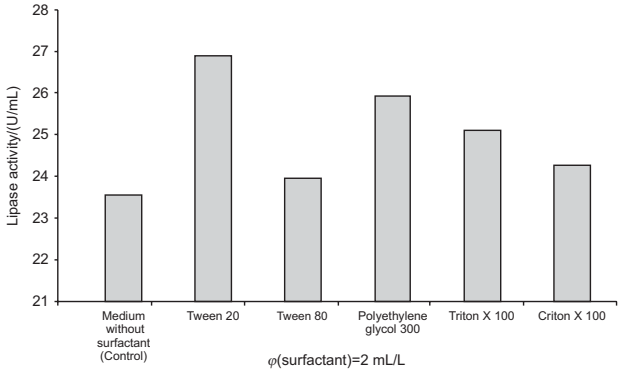

Fig. 5. Influence of different surfactants on lipase production

production in *S. rubidaea* depended on their fraction. Among the tested fractions of Tween 20 from 3.0 to 10.0 mL/L, at an interval of 1 mL/L, the lipase production was maximum ((34.20±0.01) U/mL) in the medium with 6.0 mL/L. The lipase activity was low ((30.93±0.04) to (32.66±0.03) U/mL and (30.46±0.045) to (33.53±0.013) U/mL) in medium supplemented with 3.0 to 5.0 and 7.0 to 10.0 mL/L of Tween 20, respectively. One-way ANOVA test showed that various concentrations of Tween 20 were statistically significant ($p<0.001$).

Partial purification of lipase

The results of partial purification of lipase indicated that the enzyme activity was found to be 8-fold more than the crude enzyme, and it was recorded that the molecular mass of partially purified enzyme was 54 kDa (Fig. 6). In consistence with this study, Matsumae and Shibatani (*16*) found that the molecular mass of *S. marcescens* SR418000 purified lipase was 62 kDa, while that of *S. grimesii* lipase was 57 kDa (*15*). Abdou (*6*) also reported that the molecular mass of purified lipase of *S. marcescens* was 52 kDa. The observation of Arpigny and Jaeger (*23*) inferred that the molecular mass of lipases from *P. fluorescens* and *S. marcescens* was 50 and 65 kDa, respectively.

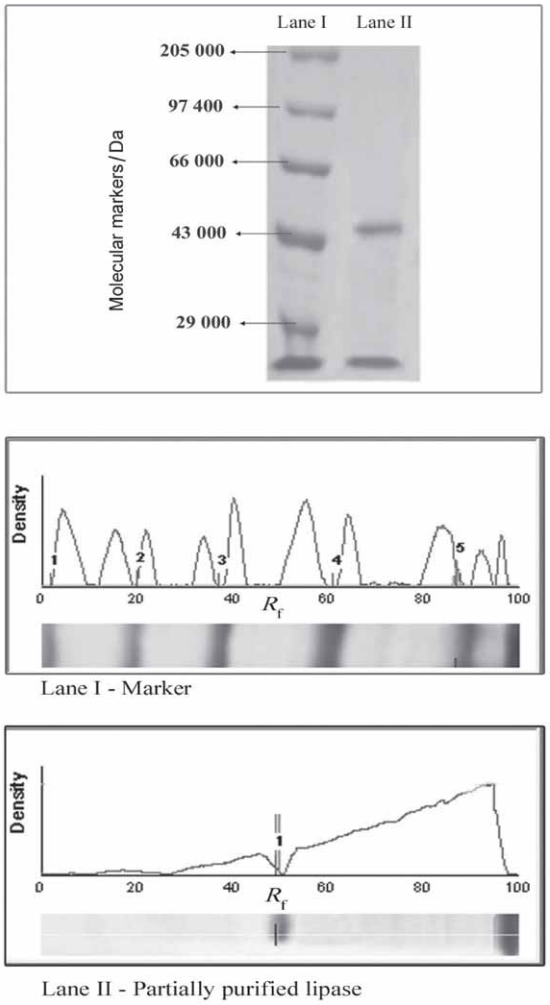

Fig. 6. SDS-PAGE profile of partially purified lipase

Conclusion

The present study revealed that extracellular lipase production by *S. rubidaea* isolated from milk samples was found to be accelerated at optimized culture conditions such as medium pH, temperature and various substrate concentrations. From the results, it could be concluded that the medium pH of 8.0 and temperature of 40 °C were optimum for maximizing lipase production by *S. rubidaea*. The assessment of various substrates for optimizing the production of lipase by *S. rubidaea* inferred that the optimum substrate contained: casein 24 g/L, soytone 5 g/L, starch 4 g/L, gingily oil 15 mL/L, Tween 20 6 mL/L. The lipase produced by *S. rubidaea* was partially purified and the molecular mass of the purified enzyme was 54 kDa. Therefore, *S. rubidaea* is a potential strain for lipase production by utilizing some of the cheapest substrates.

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