

PRODUCTION, PURIFICATION, CHARACTERIZATION AND APPLICATIONS OF LIPASE FROM *SERRATIA MARCESCENS* MBB05

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ABSTRACT

The aim of this study was to produce, purify, characterize and manufacture of cheddar cheese properties of lipase isolated from *Serratia marcescens* MBB05. The potential isolate was screened for lipase activity on Rhodamine B agar medium and the strain was studied for the cost effective production of the industrially important enzyme. For subsequent studies all the physico-chemical parameters tested, the best results were obtained in medium having defatted soybean flour at 25°C at pH 6.0. The purified enzyme was homogeneous with the estimated molecular mass of 63.7 kDa as determined by SDS-PAGE and gel filtration. The results showed that higher lipase production was obtained by inhibited protease enzyme production. Further, the lipase enzyme could be used as a future alternative to the commercial cheese flavour enhancing material and sebum degradation.

Keywords: *Serratia marcescens* MBB05, Soy bean flour, Lipase, Sebum degradation, Cheese production.

INTRODUCTION

The advent of enzymology represents an important breakthrough in the biotechnology industry, with the worldwide usage of enzymes being nearly U.S. \$ 1.5 billion in 2000¹. The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as lipases, proteases, amylases, amidases, and esterases. In recent times, lipases (triacylglycerol acylhydrolase, E.C.3.1.1.3) have emerged as key enzymes in swiftly growing biotechnology, owing to their multifaceted properties^{2, 3, 4}. Lipases or triacylglycerol acyl-ester hydrolases are carboxylesterases that catalyze both hydrolysis and synthesis of esters formed from glycerol. Sources of lipases are animal and vegetable tissues and microorganisms. Microbial enzymes are often more stable and their production is more convenient and safer. The interest in microbial lipase production has increased in the last decades, because of its large potential in a wide range of industrial applications includes processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture and production of cosmetics and pharmaceuticals^{5, 6-8}. However, in recent years, *Serratia* sp. has been studied for its ability to produce lipase. Among *Serratia* species, *S. marcescens* having the trait to produce extracellular enzymes including chitinase, several proteases, nuclease and lipase. It is a motile, gram negative, rod shaped facultative anaerobe, is reported to grow in temperatures ranging from 5 - 40°C and having the ability to form prodigiosin is one of its unique characteristics⁹⁻¹¹.

Current applications include the flavour enhancement of cheeses, the acceleration of cheese ripening, the manufacturing of cheese like products, and the lipolysis of butterfat and cream. The free fatty acids generated by the action of lipases on milk fat endow many dairy products, particularly soft cheeses, with their specific flavour characteristics¹². Lipases are used in the development of fragrances, the manufacture of host skin-care products, and in most cosmetic products. Noriko *et al.*¹³ invented a face wash, which effectively removes pore stains and aged horny layers of the skin and gives whitening effect but has lesser stimulant feel. This face washes comprising a protein-degrading enzyme, lipase, trehalose, D - glucose and ascorbic acid and/or a derivative thereof as effective ingredients. Luis¹⁴ invented a cosmetic composition for the removal of sebum exudates from the skin. The composition comprises immobilized enzymes namely lipolytic lipases, proteolytic proteases and enzymes for the breakdown of sugar oligomers from glucoproteins in the skin exudates. The enzymes may be present individually or in any desired combination. The fats from sebum tend to adsorb and absorb solids, which then form crusts and flakes. The lipase, when activated from its immobilized form, breaks down the lipid components of sebum, linear fatty acid esters, fatty acids or polycyclic cholesterol, to lower molecular

weight compounds. This work was the first article which was carried out the research in lipase production from *Serratia marcescens* MBB05. Thus, the present study was carried out for the production, purification, characterization of lipase from *Serratia marcescens* MBB05 and its application in sebum degradation and cheese production.

MATERIALS AND METHODS

Collection of organism

Serratia marcescens MBB05 culture was used for the lipase enzyme production. It was obtained from Karpagam Microbial Culture Collection Centre (KMCCC), Karpagam University, Coimbatore. This culture was maintained on nutrient agar (g/L) (peptone, 5; beef extract, 3; yeast extract, 2; sodium chloride, 5; agar, 1.5; pH 7.0 ± 0.2) slant. This culture was stored at 4°C until further use.

Screening method for lipase production

The organism was tested for lipase production on Rhodamine B agar (Peptone, 10 g; yeast extract, 3 g; sodium chloride, 5 g; bacto agar, 30 g; polyvinyl alcohol, 2%; olive oil, 5 mL; Rhodamine B, 10 mg; Distilled water, 1000 mL) plates. After 3 days of incubation at 30°C, the lipolytic activity was confirmed by the halos around the colony. An orange fluorescent halos was observed irradiating plates with UV light at 350 nm¹⁵.

Selection of medium for lipase production by submerged fermentation

Test strain of *S. marcescens* MBB05 was cultivated in 10 different media differing in the contents of mineral components for preliminary study of effect in nutrient medium composition on lipase biosynthesis. *S. marcescens* MBB05 was cultivated separately in 250 mL flasks containing 100 mL of a culture medium 1 to 5 (Medium 1: Na₂HPO₄, 0.7%; NH₄Cl, 0.1%; CaCl₂.2H₂O, 0.002%; NaCl, 0.05%; MgSO₄.7H₂O, 0.025%; KH₂PO₄, 0.3%; Decoction of non defatted soybean flour, 5%; pH 7.0. Medium 2: Urea, 0.1%; MgSO₄.7H₂O, 0.1%; KH₂PO₄, 0.2%; Decoction of non-defatted soybean flour, 5%; pH 7.0. Medium 3: (NH₄)₂SO₄, 0.1%; MgSO₄.7H₂O, 0.03%; KH₂PO₄, 0.14%; Decoction of non-defatted soybean flour, 5%; pH 7.0. Medium 4: Na₂HPO₄, 0.7%; NH₄Cl, 0.1%; CaCl₂.2H₂O, 0.002%; NaCl, 0.05%; MgSO₄.7H₂O, 0.025%; KH₂PO₄, 0.3%; Olive oil, 5%; pH 7.0. Medium 5: Na₂HPO₄, 0.7%; NH₄Cl, 0.1%; CaCl₂.2H₂O, 0.002%; NaCl, 0.05%; MgSO₄.7H₂O, 0.025%; KH₂PO₄, 0.3%; Decoction of defatted soybean flour, 5%; pH 7.0) in a shaker at 160 rpm at 30°C for 24 h¹⁶.

The lipolytic activity on the liquid medium was assessed by enriching *S. marcescens* MBB05 in the enrichment medium

containing beef extract, 0.3%; peptone, 0.5%; NaCl, 0.5%; glucose, 0.5%; pH 7.0 and then 10% of enriched culture was inoculated in 250 mL flask containing 90 mL of basal medium 6 (TS broth (g/L) tryptone, 16; soytone, 3; NaCl, 5; glucose, 2.5; K₂HPO₄, 2.5). The culture was then incubated for 24 h by reciprocal shaking at 150 rpm at 30°C¹⁷. *S. marcescens* MBB05 was inoculated into nutrient broth (g/L) peptone, 5; beef extract, 3; yeast extract, 2; sodium chloride, 5; pH 7.0 ± 0.2) and incubated at 30°C for 24 h. From that 150 µL aliquots were inoculated into 15 mL portions of sterile medium 7 (10% reconstituted skim milk in distilled water) and incubated at 30°C for 24 h¹⁰. Seed cultures were prepared by inoculating 30 mL of the medium 8 (peptone, 5 g; yeast extract, 5 g; KH₂PO₄, 9.1 g; K₂HPO₄, 5.7 g; MgSO₄, 0.5 g; NaCl, 0.5 g; FeSO₄·7H₂O, 0.01 g; Distilled water, 1000 mL; pH 6.5) in a 250 mL flask with *S. marcescens* MBB05, with subsequent incubation for 12 h at 30°C with shaking at 150 rpm. The flask culture experiments were performed at 30°C and 150 rpm for 24 h in 250 mL flask containing 100 mL medium, inoculated with 2.5% (v/v) of the seed culture¹⁸.

Hundred milliliters of the medium 9 (g/L) (peptone, 30; Tween 80, 10; K₂HPO₄·3H₂O, 7.47; NaH₂PO₄·2H₂O, 10.43; MgSO₄, 0.5; NaCl, 0.5; FeSO₄·3H₂O, 0.01) was transferred into 250 mL flask and then inoculated with the *S. marcescens* MBB05. Then the microbial fermentation was carried out at 200 rpm, 30°C for 24 h¹⁹. The flask containing 100 mL of medium 10 (nutrient broth (g/L) peptone, 5; beef extract, 3; yeast extract, 2; sodium chloride, 5; pH 7.0 ± 0.2) was inoculated with *S. marcescens* MBB05 and incubated at 30°C for 24 h²⁰. After incubation period, all the fermented broths were subjected to centrifuge for removal of cells at 10,000 rpm for 15 min at 4°C, and the supernatant fluids were assayed for exolipase activity.

Lipase assay method

Lipase was assayed using olive oil as the substrate²¹. The substrate was prepared by mixing the 2 mL of olive oil with 25 mL of water in the presence of 100 mg bile salts and stirred well until an emulsion is formed. Then 2 g of gum arabic was added to hasten emulsification. From the substrate mixture, 20 mL was added to 5 mL of 50 mM phosphate buffer pH 7.0 and stirred the contents slowly by keeping on top of a magnetic stirrer cum hot plate at 35°C. The electrode of a pH meter was dipped in the reaction mixture and the pH was adjusted to 7.0. Then 1 mL of lipase enzyme was added and immediately recorded the pH at zero min. Then at frequent intervals (say 10 min) or as the pH drops by about 0.2 units, 0.1 N NaOH was added to bring the pH to the initial value. This was continued for 30 min period and the volume of alkali consumed was recorded. Then the lipase activity was calculated according to the formula described in Sadasivam and Manickam²².

Lipase activity
 $(\mu\text{g}/\text{min}/\text{mL}) = \frac{\text{Volume of alkali consumed} \times \text{Strength of alkali} \times 1000}{\text{Volume of sample} \times \text{Time in min}}$

One unit (U) of lipase activity is equal to one µmol of free fatty acid liberated per min per mL using the assay condition.

Protein estimation

Quantitative estimation of the protein content was done by Lowry *et al.*²³ method.

Optimization of various physico-chemical parameters

The extracellular lipase produced by *S. marcescens* MBB05 was first examined by cultivation of the bacteria in selected media. This prompted to optimize various parameters to improve production of bacterial lipase.

Effect of temperature

Investigations on the effect of cultivation temperatures on lipase production have been carried out by incubating the medium 5 at different temperatures. The lipase production was carried out at 20, 25 and 30°C keeping all other conditions at their standard

levels and then assayed for lipase. The optimum temperature achieved by this step was fixed for subsequent experiments¹⁸.

Effect of pH

In order to study the effect of pH of the medium 5 on lipase production, experiments were performed with medium of different pH. While optimizing the pH of the basal medium, the pH of aqueous solution was varied from 5.5 to 8.0 with 0.1 M NaOH or 0.1 N HCl and then assayed for lipase. The optimum pH achieved by this step was fixed for subsequent experiments¹⁸.

Effect of incubation time

To determine the optimum incubation period for lipase production, medium 5 were incubated for different time durations (20, 22, 24, 26 and 28 h) and then assayed for lipase. The optimum incubation period achieved by this step was fixed for subsequent experiments¹⁶.

Effect of inoculum size

To evaluate the effect of inoculum size on lipase production varied cell concentrations (1.5, 2.0, 2.5, 3.0 and 3.5%) were added to flask containing medium 5 and then assayed for lipase production. The fermentation was carried out at 30°C keeping all other conditions at their optimum levels. The optimum inoculum level achieved by this step was fixed for subsequent experiments¹⁸.

Effect of nitrogen source

Different nitrogen sources such as peptone and yeast extract (1:1), peptone and yeast extract were supplemented separately to a final concentration of 5 g/L in the medium 5. After incubation in an optimal condition, the lipase was studied¹⁸.

Effect of carbon source

Different carbon sources such as olive oil, glucose, glycerol, and tween 80 were supplemented separately to a final concentration of 0.5% (v/v) in the medium 5. After incubation in an optimal condition, the lipase was quantified¹⁸.

Effect of metal ions

Different metal ions such as 1 mM KCl, 10 mM CaCl₂, 1 mM NaCl and 1 mM MgSO₄ were supplemented separately in the medium 5. After incubation in an optimal condition the lipase was studied.

Effect of medium components

The optimizations of medium components were carried out by substituting the medium components at various concentrations in the medium 5. After incubation in an optimal condition, the lipase was quantified.

Protease assay

Protease assay was performed as reported by Jayaraman *et al.*²⁴. 1 mL of casein solution with 0.2 mL of enzyme solution was incubated at 37°C for 1 h. Then 1 mL of 12% trichloro acetic acid solution was added and the mixture was cooled rapidly in an ice. It was centrifuged and the supernatant was collected separately. Then 5 mL of 40% formaldehyde was added to the supernatant, followed by a few drops of phenolphthalein indicator. It was titrated against 0.1 N sodium hydroxide until a faint but permanent pink colour was formed. Then the end point was noted and the amount of sodium hydroxide added to neutralize was calculated. This is a measure of the free amino groups produced by the liberated amino acids.

Inhibition of protease enzyme

The protease enzyme was inhibited in the culture supernatant by adding the EDTA at various percentages such as 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0. Then the protease activity was tested for all. Simultaneously the lipase activity was also tested to select the best one²⁵.

Production of lipase enzyme

The final production of lipase enzyme was done by culturing the organisms in the desired medium with optimized conditions. After culturing, the culture supernatant was collected and protease enzyme was inhibited. Then the lipase activity was tested and confirmed the highest production of lipase.

Purification of lipase enzyme

Crude lipase enzyme preparation

Culture supernatant containing crude lipase enzyme was obtained by centrifugation of the culture broth of *S. marcescens* MBB05 at 10,000 rpm for 20 min. The lipase assay and protein estimation was performed to crude enzyme as described previously.

Ammonium sulphate fractionation

The enzyme from the cell free supernatant was precipitated by ammonium sulphate at 80% saturation and kept overnight at 4°C. The precipitate was collected by centrifugation at 15,000 rpm for 15 min and was dissolved in a minimum quantity of 10 mM Tris-HCl buffer (pH 7.5). Then the lipase assay and protein estimation was done to this sample as described previously^{26,20}.

Dialysis

The precipitated solution was dialyzed against the same buffer (10 mM Tris-HCl buffer, pH 7.5) overnight at 4°C by using dialysis membrane -50 with flat width of 24.26 mm, diameter of 14.3 mm and capacity 1 mL/cm. The lipase assay and protein estimation was performed to dialyzed sample as described previously²⁶.

Column chromatography

The dialyzed solution containing both soluble and insoluble material was put together on cellulose (C₆M₁₀O₅)_n powder column (2.5 × 15 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.5. The column was washed with four bed volumes of the same buffer. The sample was eluted with a linear NaCl gradient (2 L), from 0 to 0.8 M, in the same buffer. The flow rate was adjusted to 0.5 mL/min (fraction volume: 5 mL). The lipase assay and protein estimation was performed to each fraction as described previously. The lipase rich fractions were pooled, stored at 4°C, and used as the purified lipase²⁶.

Determination of molecular weight by SDS PAGE

The molecular weight of the lipase purified from column chromatography was checked by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE). It was performed as described by Laemmli *et al.*²⁷.

Characterization of lipase enzyme

Effect of pH on the activity and stability of lipase

The activity of lipase was examined within the pH range of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 using the following buffer solutions of 0.1 M citrate phosphate buffer- pH, (3.5 - 6.0); 0.1 M sodium phosphate buffer - pH, (6.5 - 7.5); 0.1 M tris HCl buffer - pH, (8.0 - 8.5); 0.1 M carbonate bicarbonate buffer pH 9.0 with olive oil as substrate. The effect of pH on lipase stability was determined by incubating the lipase in desired buffer and desired pH for 30, 40, 50, 60, 70, 80 and 90 min respectively. After the incubation period, 100 µL aliquots of the buffered enzyme solutions were taken and the lipase activity was assayed as described previously²⁸.

Effect of temperature on the activity and stability of lipase

The activity of lipase was examined within the temperature range (°C) of 20, 30, 40, 50, 60, 70 and 80°C with olive oil as substrate. The effect of temperature on lipase stability was determined by incubating the lipase in desired temperature for 30, 40, 50, 60, 70, 80 and 90 min respectively. After the incubation period, the enzyme was rapidly cooled and the lipase activity was assayed as described previously⁵.

Application of lipase enzyme on sebum degradation

Sebum collection

Samples of the sebum were obtained from the cheek of healthy adult. To collect the sample, a strip of filter paper (Whatman No.1, 3 cm X 12 cm), previously defatted by extraction with boiling light petroleum, was placed on the skin of the cheek lightly cleaned by a cotton wad soaked in ethyl ether. The paper was attached to the skin and kept there for 2 h, then replaced by a new one for another 2 h. The strips of paper with samples of sebum were kept at 4°C, then on the same day extracted with 20 mL of boiling chloroform - methanol (4:1, v/v). The extract, after removal of the solvents, was weighed and stored at 4°C.

Sebum analysis

The sebum sample was dissolved in the above mentioned mixture of chloroform - methanol, at a concentration of 1 mg / 0.2 mL. About 30 µL of this solution was chromatographed on a whatman No. 1 paper, then eluted first by chloroform - methanol mixture to a distance of 0.8 cm, then by acetone to 1.5 cm and finally by methanol to 2.5 cm. Before addition of each further eluent, the chromatogram was dried. When separation was finished the disk chromatogram was treated separately by different reagents for detection of individual components. For the detection of cholesterol, acetic and sulphuric acid and for amino lipid, ninhydrin was used. In addition, free acids were detected by the reduction of their silver salts. The chromatogram was immersed for 3 min in 10% AgNO₃, then rinsed thoroughly in water and dried. The presences of free acids were detected by a dark brown colour. For choline lipid, the chromatogram was treated with potassium dichromate in 1% ethanolic solution of diphenylcarbazide or haematoxylin Diphenylcarbazide gave immediately a red - violet stain, which, however, was soluble in water, so that the chromatogram was evaluated very rapidly²⁹.

Analysis of sebum degradation by lipase enzyme

The lipase assay method was performed with sebum as substrate instead of olive oil. 40 µg of sebum was taken for analysis of degradation by lipase enzyme. 83 U/mL of purified lipase from *S. marcescens* MBB05 was used for assay. After performing the assay, the amount of fatty acid liberated per min per mL was calculated as described previously.

Application of lipase on cheese production

Cheeses were prepared according to the procedure as described previously by Sukumar de *et al.*³⁰. The cheese using bacterial lipase of *S. marcescens* MBB05 were produced using cow milk. 100 mL of raw milk from a nearby farm was collected, pasteurized at 72°C for 15 seconds and cooled to 32-35°C. After 1 h of milk ripening at 30°C, the 10 mL of pure bacterial lipase was added to clot the milk in 40 min. Following coagulation, the whey was drained out. Then it was salted (3%, w/w, NaCl) by spreading coarse salt over the surfaces manually and pressed. Then the cheeses were stored in a ripening chamber with a relative humidity of 82% and at a temperature of 12°C. After that, the cheese was characterized for its taste, color, texture, and odor, finally it was then stored at 4°C.

RESULTS AND DISCUSSION

Screening and selection of medium for lipase production by submerged fermentation

The lipolytic activity of *S. marcescens* MBB05 were observed by the formation of halo around the colonies upon hydrolysis of olive oil by the enzyme lipase. Cultivation in medium 5 resulted in higher lipolytic activities for *S. marcescens* MBB05. 1 U/mL of lipase activity was obtained for *S. marcescens* MBB05 (Fig. 1). The nutrient media composition strongly affected the biosynthesis of the enzyme. Both the mineral component and the fat content of the soybean flour were important. Duzhak *et al.*¹⁶ reported 70, 720, 840 AU/mL of lipase activity in media 1, 2 and 5 respectively for *S. marcescens* strain V - 10.

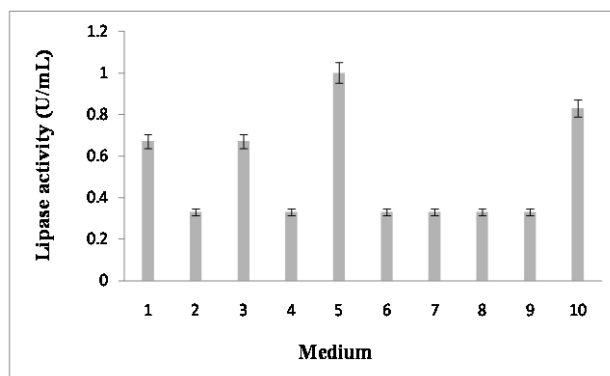


Fig 1: Effect of medium components on Lipase production by *Serratia marcescens* MBB05 (pH, 7.0; incubation time, 24 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL

Optimization of various physico - chemical parameters

Effect of temperature

Experiment on the effect of temperature indicated that the lipase production of *S. marcescens* MBB05 was maximum 1 U/mL at the

temperature of 25°C. However, the low activity of lipase was observed at 20 and 30°C (Fig. 2). Gao *et al.*¹⁸ reported the protease accumulation at 30°C was responsible for low activity.

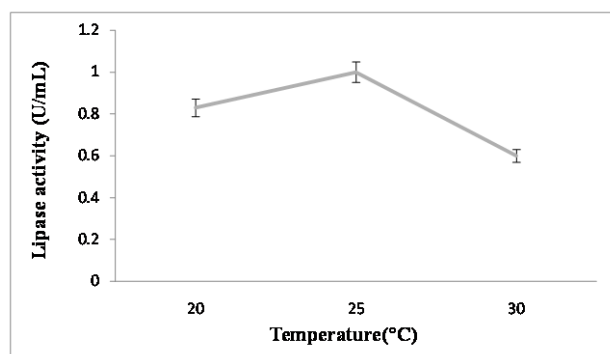


Fig 2: Effect of temperature on Lipase production by *Serratia marcescens* MBB05 (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

Effect of pH

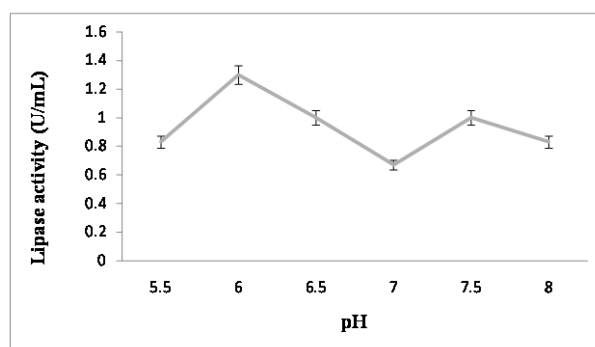


Fig 3: Effect of pH on Lipase production by *Serratia marcescens* MBB05 (Incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

The effect of the pH of the medium on lipase production by *S. marcescens* MBB05 indicated a maximum lipase production 1.3 U/mL at pH 6.0 was observed (Fig. 3). Gao *et al.*¹⁸ reported this strain of bacterium prefers a slightly acidic pH for lipase production, and these conditions were also suitable for the stabilization and accumulation of extracellular lipase.

Effect of incubation time

The influence of incubation time on lipase production revealed that maximum lipase production 0.5 U/mL for *S. marcescens* MBB05 upon the incubation at 20 h (Fig. 4). The time of incubation increased for these cultures showed sharp decrease in lipase activity. The very low lipase activity of 0.16 for *S. marcescens* MBB05 was observed at 28 h of incubation. Duzhak *et al.*¹⁶ reported the lipase production by strain occurred at the end of the logarithmic growth phase after 10-5 h of cultivation is responsible for maximum lipase production at 20 h.

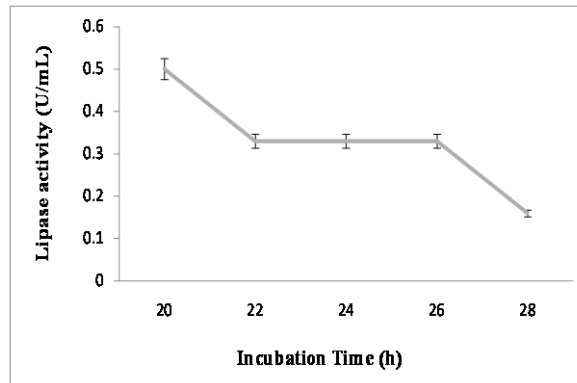


Fig 4: Effect of incubation time (h) on Lipase production by *Serratia marcescens* MBB05 (pH, 6.0; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

Effect of inoculum size

The effect of inoculum size resulted that the higher lipase activity by *S. marcescens* MBB05 (0.67 U/mL) was observed at 2.5% (Fig. 5). The low activity was observed at 1.5% in *S. marcescens* MBB05. This optimized inoculum size was maintained for further study.

Gao *et al.*¹⁸ reported that the strain of *S. marcescens* ECU1010 produced 500 U/L of extracellular lipase activity by using the 2.5% (v/v) of the seed culture.

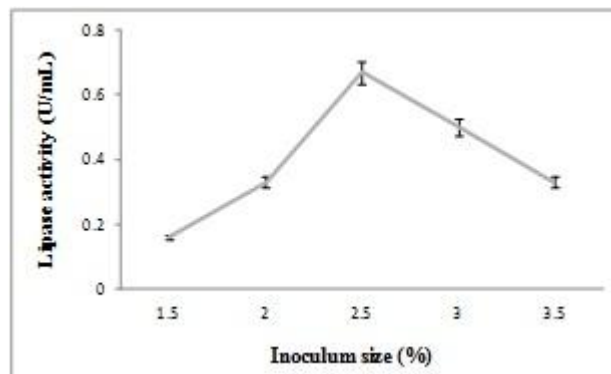


Fig 5: Effect of inoculum size (%) on Lipase production by *Serratia marcescens* MBB05 (pH, 6.0; and incubation time, 20 h) results are mean of independent experiments \pm SD and are expressed as U/mL.

Effect of nitrogen source

Different nitrogen sources were tested at a fixed concentration (5 g/L). Both the cell growth and enzyme activity were greatly affected. Among the nitrogen sources, the enzyme activity was low about 0.16 U/mL by *S. marcescens* MBB05 on yeast extract. *S. marcescens* MBB05 showed the higher activity of 0.33 U/mL at peptone alone (Fig. 6). Gao *et al.*¹⁸ reported the yeast extract is a

complex nitrogen source and thus requires the cells to secrete more protease for its enzymatic degradation before utilization. This would result in less production and more degradation of the extracellular lipase. Since *S. marcescens* produces an extracellular lipase and the total enzyme activity is more important for practical application, so peptone was selected as the optimal nitrogen source due to its larger capacity of supporting the lipase production.

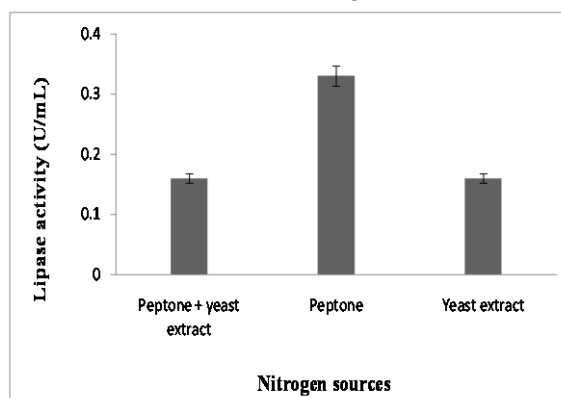


Fig 6: Effect of nitrogen sources on Lipase production by *Serratia marcescens* MBB05 (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

Effect of carbon source

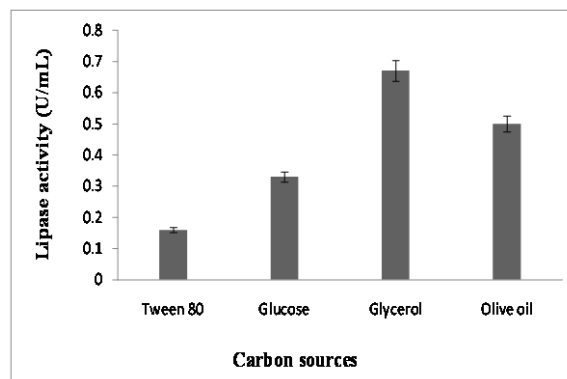


Fig 7: Effect of carbon sources on Lipase production by *Serratia marcescens* MBB05 (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

On the basis of the activity assay, it was concluded that better growth and higher activity was obtained on medium supplemented with glucose and glycerol. The higher lipase activity of 0.67 U/mL for *S. marcescens* MBB05 was observed on medium supplemented with glycerol (Fig. 7). Low enzyme activity was obtained with Tween 80 and olive oil. Tween-80 supported cell growth but yielding the lower lipase activity. Samad *et al.*³¹ reported that the glycerol showed stimulatory effects when comparing to other carbon sources.

Effect of metal ions

The effect of different metal salts on lipase activity was revealed that the 1 mM NaCl stimulated the higher lipase activity of about 0.5 U/mL on *S. marcescens* MBB05 (Fig. 8). The low lipase activity about 0.16 U/mL for *S. marcescens* MBB05 was observed on 1 mM MgSO₄.

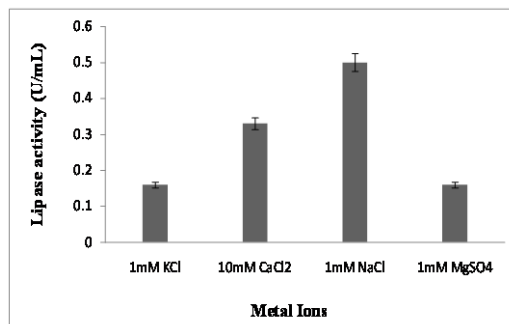


Fig 8: Effect of metal ions on Lipase production by *Serratia marcescens* MBB05 (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

Effect of medium components

The effect of medium components reported that the higher lipase activity of about 0.5 U/mL was observed on *S. marcescens* MBB05 when double the amount of KH₂PO₄ (Fig. 9). However, the Na₂HPO₄ showed very low activity on *S. marcescens* MBB05. The presence of potassium stimulated the lipase activity. Winkler and Stuckmann³² reported that the presence of sodium, potassium, calcium in pectin B stimulates the lipase activity.

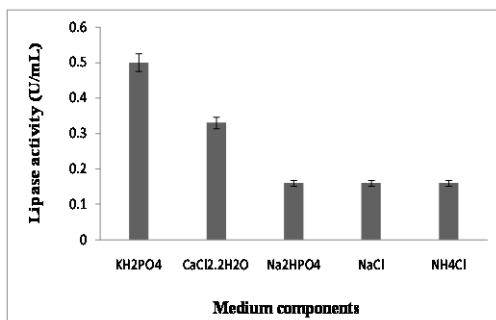


Fig 9: Effect of medium components on Lipase production by *Serratia marcescens* MBB05 (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

Protease assay

The protease assay resulted that there was very higher production of extracellular protease by *S. marcescens* MBB05. It showed about 30 U/mL of protease activity when comparing to lipase production (Fig. 10).

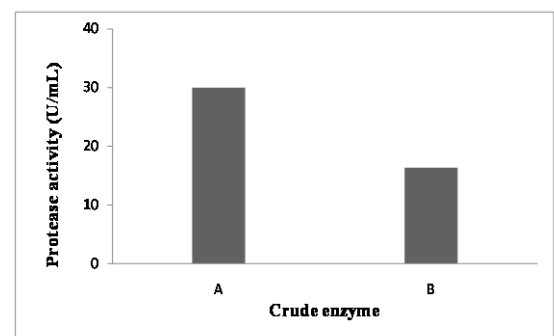


Fig 10: Protease activity of *S. marcescens* MBB05 in crude enzyme (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL (A - Before Inhibition of protease enzyme, B - After Inhibition of protease enzyme).

It was nearly 25 - 30 times higher than lipase production. There was nearly 50% decreased protease production was observed after the inhibition of protease activity in crude enzyme by using 0.2% EDTA in culture supernatant of *S. marcescens* MBB05. Thus, the crude enzyme was treated to inhibit the protease enzyme in all other studies. Bayoumi *et al.*³³ reported that a sharp decrease in lipase production occurred after 72 h of cultivation which could be due to proteolytic degradation of enzyme system.

Production and purification of lipase enzyme

The production of lipase by *S. marcescens* MBB05 showed 1.0 U/mL before optimization of various parameters in crude enzyme. The amount of lipase production was slightly increased about 1.83 U/mL by *S. marcescens* MBB05 after optimizing the various parameters (Fig. 11). Still it was very low due to the degradation of lipase by protease enzyme.

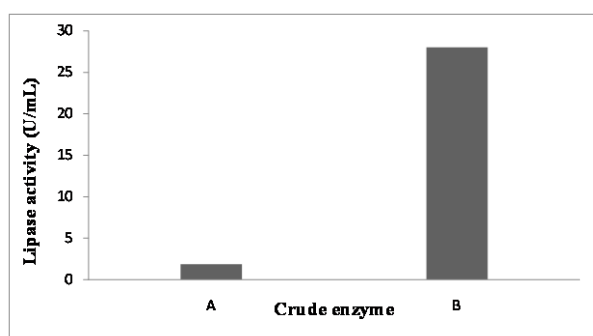


Fig 11: Lipase activity of *S. marcescens* MBB05 in crude enzyme (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL (A - Before Inhibition of protease enzyme, B - After Inhibition of protease enzyme).

Table 1: Purification of lipase from *S. marcescens* MBB05

Purification steps	Total volume (mL)	Lipase activity (U/mL)	Protein content (μ g/mL)	Total activity	Specific activity	Purification fold	Recovery (%)
Crude enzyme	50	28	70	1400	0.4	1	100
Ammonium sulphate precipitation	15	46.67	58	700	0.8	2	50
Dialysis	10	66.66	46	666.6	1.44	3.6	47.61
Column chromatography	5	83.33	24	416.65	3.47	8.67	29.76

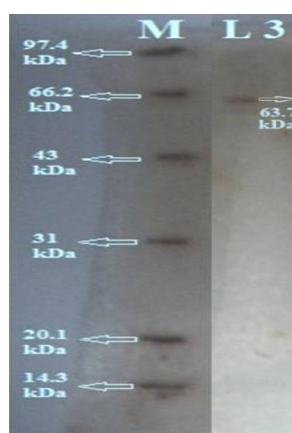


Fig 12: SDS-PAGE profile of purified lipase by *S. marcescens* MBB05. Lane M represents protein marker (14.3 - 97.4 kDa), Lane 3 represents purified lipase (63.7 kDa).

However, the lipase production was increased to very higher about 28 U/mL upon inhibition of protease enzyme by EDTA at the concentration of 0.2% in the crude enzyme of *S. marcescens* MBB05. The EDTA has the ability in degrading the protease enzyme, which leads to the decrease in the degradation of lipase by protease enzyme. The purification of lipase was performed by 3 steps. The lipase activity on each purification step was continuously increased in lipase of *S. marcescens* MBB05. The column fractions of 19 - 33 were showed higher lipase activity (Table 1), among this 26th fraction showed very higher lipase activity in *S. marcescens* MBB05. There was 8.67 times purification fold was obtained for *S. marcescens* MBB05 lipase. The recovery of 29.76% lipase was obtained from *S. marcescens* MBB05.

Determination of molecular weight by SDS PAGE

SDS PAGE revealed the presence of a single band in lane 3 (Fig. 12) with a molecular weight of 63.7 kDa by comparing with standard protein molecular marker with known molecular weight standard (14.3 - 97.4 kDa) (97.4 kDa, phosphorylase B; 66.2 kDa, bovine serum albumin; 43 kDa, ovalbumin; 31 kDa, carbonic anhydrase; 20.1 kDa, trypsin inhibitor; 14.3 kDa, lysozyme). It was indicated that the molecular weight of *S. marcescens* MBB05 Lipase was 63.7 kDa. Zhao *et al.*³⁴ reported that the molecular mass of 65 kDa is a relatively high value among other known lipases from bacteria. Yet, *S. marcescens* strains are known to produce lipases that have relatively large molecular masses, ranging from 52 to 65 kDa.

Characterization of lipase enzyme

Effect of pH on the activity and stability of lipase

The purified lipase hydrolyzed olive oil as substrates appreciably over a relatively broad pH range from pH 3.5 to 9.0. *S. marcescens* MBB05 Lipase had shown maximum activity at pH 7.0 (Fig. 13 and 14). At alkaline pH, the activity of the enzymes decreased gradually and at acidic pH, the slight decrease was observed. In addition, the *S. marcescens* MBB05 Lipase was found to be stable for 40 min at pH 7.0.

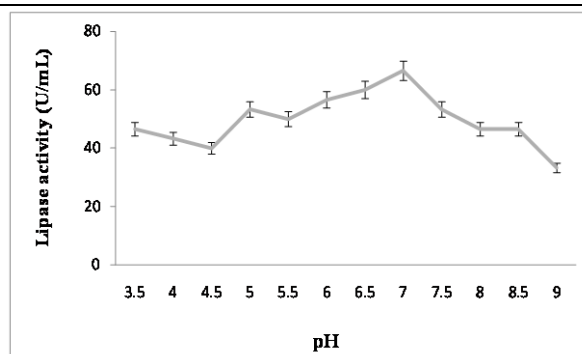


Fig 13: Activity of lipase at different pH of *S. marcescens* MBB05 in crude enzyme (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

Gao *et al.*¹⁸ reported that the stability of the crude enzyme in the culture medium was shown to decrease with either increasing or decreasing pH values. The enzyme was relatively stable at pH 6.5 to 7.0, with 90% (at pH 6.5) or 92% (at pH 7.0) of the initial

activity remaining after 4 h at 30°C. The enzyme activity decreased more sharply at high pH than at low pH, the residual activity being 79% at pH 5.0, but only 57% at pH 9.0, and 28% at pH 9.5. The reason is degradation of the lipase protein by proteases, which may be more active at pH > 7.0.

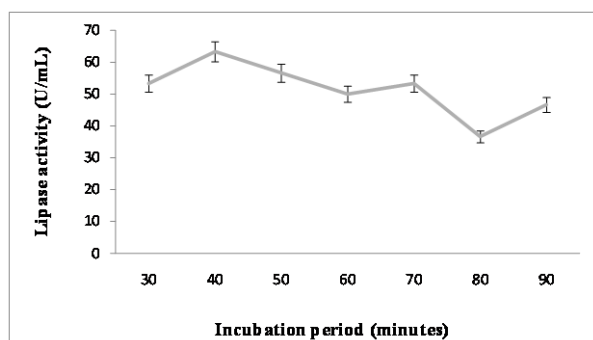


Fig 14: Stability of lipase at pH 7.0 of *S. marcescens* MBB05 in crude enzyme (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

Effect of temperature on the activity and stability of lipase

The effect of temperature on the activity of *S. marcescens* MBB05 Lipase was examined in the range of 20 to 80°C. Lipolytic

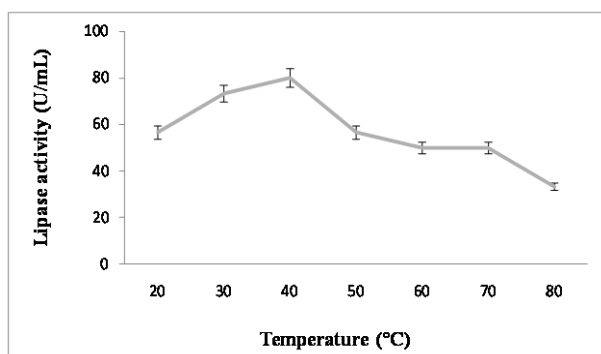


Fig 15: Activity of lipase at different temperature of *S. marcescens* MBB05 in crude enzyme (pH, 6.0; incubation time, 20 h; and inoculum, 2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

activity of this lipase was found to be profoundly affected by temperature. The activity of lipase was increased gradually with rise in temperature up to 40°C and the maximum activity was observed at 40°C (Fig. 15 and 16). However, the temperature of 50 and 60°C showed nearly 85% of activity.

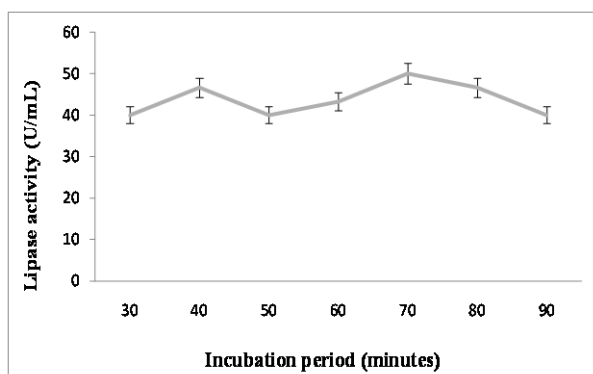


Fig 16: Stability of lipase at 40°C of *S. marcescens* MBB05 in crude enzyme (pH, 6.0; incubation time, 20 h; and inoculum,

2.5%) results are mean of independent experiments \pm SD and are expressed as U/mL.

The thermal stability of purified lipase was studied at 40°C. Lipase had maximum stability for 70 min. The 50% stability was obtained at 80 min of incubation at 40°C. The results revealed that the lipase was heat-stable. Roy³⁵ suggested that *S. marcescens* and *P. fluorescens* lipases are probably more stable at low temperatures.

Application studies of lipase enzyme

Sebum collection

The sample of sebum was collected about the average of 200 mg. i.e. 5.56 mg/cm². Horacek and Marie cernikova²⁹ reported that the average of 21.5 mg of sebum was obtained, i.e. 0.596 mg/cm².

Cheese production and analysis of sebum degradation by lipase enzyme

The lipase from *S. marcescens* MBB05 of about 83 U/mL was used for the degradation of 40 μ g of sebum. Upon hydrolysis of sebum by lipase, 15.33 μ g/min/mL of fatty acid were released. Noriko *et al.*¹³ reported that the use of any lipases, usually, in an amount of 0.01 unit or more, desirably, 0.1 to 100,000 units, more desirably, 1 to 10,000 units, most desirably, 10 to 1000 units per one gram of the face wash. The cheese was prepared by using *S. marcescens* MBB05 Lipase. The bright and clear creamy white color, smooth and velvety texture, mildly salted taste and fresh skim milk odor cheese was obtained.

CONCLUSION

Microbial lipase being an industrial enzyme has got many applications in the synthesis of industrially important products such as fatty acid methyl esters, flavor esters, and enantiopure compounds and versatile biocatalysts in the pharmaceutical, food, biofuel, oleaginous, cosmetic, detergent, leather, textile, and paper industries. In this work, low-cost defatted soybean flour and non-defatted soybean flour for the production of lipase enzyme from *Serratia marcescens* MBB05 was investigated. The results showed that higher enzyme production was obtained by using defatted soybean flour under submerged fermentation method. Among all the physico-chemical parameters tested, the best results were obtained at 25°C at pH 6.0. This study also showed that the production of lipase from *Serratia marcescens* MBB05 using defatted soybean flour could be a cost effective method of enzyme production. The purified lipase enzyme was successfully applied in the manufacture of a cheddar cheese and for the degradation of sebum. Further, the lipase enzyme could be used as a future alternative to the commercial cheese flavour enhancing material and sebum degradation.

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