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A Study on the Culture Variables Affecting the Production of Extracellular Lipase by *Serratia marcescens* VT1

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A B S T R A C T

Lipase with good activity and unique properties is significant due to its wide commercial applications in various industries. Microbes especially bacteria, fungi, and yeast inhabiting diverse habitats can form the major sources of lipase with unique properties. Growth and secretion of enzymes by any organism depend on many factors which can be nutritional, physical and chemical. The proposed work is focused on understanding the influence of various carbon and nitrogen sources, substrates, pH, agitation speed/aeration, and inoculum size on the secretion of extracellular lipase by the newly isolated bacterium *Serratia marcescens* VT 1. Sucrose (12.49 U/ml), casein (16.08 U/ml), and olive oil (24.11 U/ml) were identified as the promising carbon source, nitrogen source, and substrate for lipase induction. Nitrogen sources like sodium nitrate, potassium nitrate, urea, and thiourea were inhibitive on lipase production. Neutral pH (23.63 U/ml) and slightly basic pH (21.99 U/ml) were most effective, while agitation speeds of 120 and 150 rpm (23.01-23.41 U/ml) were efficient for lipase production. The different inoculum sizes used in the study were found to have the least effect on lipase secretion. The results from the preliminary study are useful for further optimization studies using statistical tools.

***Key words*:** Lipase, *Serratia marcescens*, Olive oil, Paranitrophenyl palmitate, Casein

Triacylglycerol acylhydrolases (EC 3.1.1.3); lipase are α/β hydrolases known for their characteristic properties like esterification, transesterification, and interesterification and unique structural features that aids in the interfacial activation occurring at the lipid water interface [21]. They constitute the third most important group of industrial biocatalyst with applications as medicines, food additives, detergent additives, clinical reagents, biodegradation, and bioremediation agents [24], [12], [10]. Lipases are omnipresent, and the major sources of commercial lipases are bacteria, fungi, and yeast. Lipase generating strains can be acquired from different habitats with varying degree of environmental conditions, and easiest way is to isolate one from a lipid polluted location as the chance for finding lipase secreting strains is much higher. The lipase generation by a particular strain can be improved by optimizing the various requirements for enzyme secretion. The concentration and type of carbon and nitrogen sources, medium pH, temperature, and dissolved oxygen concentration is known to effect lipase production [3]. The improvement of lipase production depends on a productive strain, optimized production media and fermentative conditions [22].

Lipase being an inducible enzyme [16] there production is dependent on many factors; the major ones are nutritional factors and physicochemical factors. Nutritional factors involve various nutrients which enhance microbial growth and stimulate the enzyme secretion. These nutrients can be divided into organic and inorganic nutrients depending on the origin. The major nutrients involved are carbon source and nitrogen source accompanied by other mineral salts. Carbon sources and nitrogen sources are most essential for microbial growth and enzyme production. Major carbon sources include glucose, fructose, maltose, lactose, sucrose, galactose, starch, carboxymethylcellulose (CMC). When it comes to lipase the substrates which are used for stimulating enzyme production are also mentioned as lipidic carbon sources. These substrates include various oils, triglycerides, and lipid sources. Major nitrogen sources include yeast extract, peptone, beef extract, tryptone, casein, and ammonium salts [24]. Nitrogen sources like yeast extract, peptone, beef extract can be considered as composite nutrient sources as they include other vitamins, minerals, and nutrients also. Mineral salts are essential for the growth and multiplication of organisms. They are involved in the production and enhancement of enzymes and are known to promote and inhibit enzyme activity. The common minerals include potassium, sodium, magnesium, calcium, and iron. Different salts of these elements are used in the medium for growth promotion.

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Major physicochemical factors involved are temperature, pH, oxygen concentration/ agitation speed, incubation time and inoculums size. Every organism has specific temperature and pH requirement for growth. The temperature can be adjusted accordingly using special temperature controlling incubators. These are handy in culturing and growing organisms from extreme conditions; the extremophiles. The pH of the medium can be adjusted to meet the needs before starting the culture. The oxygen concentration and availability can be controlled by using rotary shakers. Anaerobic conditions are maintained by using anaerobic fermenters. Production and secretion of enzymes by organisms are also depended on certain stimulators / substrates. These compounds are known to induce enzyme production and maintained in higher concentration compared to rest of the medium composition. The substrates are used as major energy source and the organisms will produce specific enzymes in response to degrade it. The most common substrate (lipid sources) used for lipase production is olive oil. Other substrates include glycerol, coconut oil, castor oil, fish oil etc.

Optimization of medium is very much necessary for overproduction of commercially important enzymes [8], [13]. It helps to produce maximum enzyme with best activity at minimal expense. Conventional methods involve studying a single factor at a time keeping rest of the composition constant and later formulating a new medium with the best factors resulting in good production and better activity. This method is quite time consuming and not very accurate [8]. Modern methods involve use statistical approach using computational software's which helps to lower the expense and time consumption, at the same time improve the accuracy of the process. Use of experimental designs helps to minimize the errors during optimization by determining the effect of the different parameters involved in an economical manner [1]. Even though conventional techniques have many cons over modern techniques it can be effectively used for medium formulation, which can be further optimized and improved using computational techniques. The present study focuses on the preliminary optimization of medium by studying the influence of different nutritional and physicochemical parameters involved in the lipase production by a newly isolated strain *Serratia marcescens* VT 1 (SMVT 1) as the major part affecting the production of industrially important enzymes depends on the cost of fermentation medium.

MATERIALS AND METHODS

Microorganism and its maintenance

The microorganism used in the study is SMVT 1. It was isolated from oil polluted sample collected from Althara Devi temple of Trivandrum district. The strain was maintained in nutrient agar plates supplemented with olive oil. The culture was incubated at 37°C and later stored at 4°C in refrigerator. The strain was identified by 16S rRNA sequencing, MALDI-TOF and morphological characters. Lipase activity was previously determined qualitatively by plate assays and quantitatively by spectrophotometric method.

Culture medium for submerged fermentation and effect of incubation time

Four different liquid medium were used for submerged fermentation and enzyme production study. The different media used were:

(1) Yeast extract- 0.1 g, NaCl- 0.25 g, $MgSO_4.7H_2O$- 0.05 g, $CaCl_2$. $2H_2O$- 0.01 g, K_2HPO_4- 0.07 g, KH_2PO_4- 0.03g, and olive oil- 1 g, pH- 7

(2) Peptone- 0.5 g, yeast extract- 0.5 g, NaCl- 0.5 g, olive oil- 1 ml, distilled water- 100 ml and pH- 7,

(3) Yeast extract 0.3 g, $CaCl_2.2H_2O$- 0.2 g, $MgSO_4.7H_2O$- 0.,1 g, $FeCl_3.6H_2O$- 0.04 g, and olive oil- 1ml, pH- 7, and

(4) Glucose- 0.5 g, Peptone- 0.5 g, yeast extract- 0.5 g, NaCl- 0.25 g, $MgSO_4.7H_2O$- 0.05 g and olive oil- 1ml, pH- 7. Best media among the four was used for further study (basic medium).

The effect of incubation time on lipase production was studied by incubating the culture media for a period of 120 hrs. The samples were collected at a regular interval of 24 h and lipase activity was estimated.

Effect of nitrogen sources

Thirteen different nitrogen sources were tested for optimizing the medium. One nitrogen source (0.5 g) was used at a time; rest of the media composition was maintained as such. The different nitrogen sources used were organic: peptone, yeast extract, casein, beef extract, tryptone, gelatin, and inorganic: ammonium sulphate, urea, thiourea, sodium nitrate, potassium nitrate, ammonium chloride, ammonium acetate. A medium without any nitrogen source was used as control.

Effect of carbon sources

Ten different carbon sources were analyzed for optimizing the enzyme production. The carbon sources used were glucose, CMC, sucrose, lactose, maltose, fructose, starch, xylan, xylose, and glycerol. One carbon source (0.5 g or ml) was used at a time keeping the media composition constant. Control used was the basic medium.

Effect of different substrates

The different substrates used for inducing lipase production were olive oil, sunflower oil, coconut oil, ghee, mustard oil, palm oil, sesame oil, and tributyrin. One substrate (1 ml) was used at a time and rest of the medium composition was kept same, one medium without any substrate was used as control.

Effect of best nitrogen source, carbon source, and substrate

Based on the study the best nitrogen source, carbon source, and substrate were identified. Six different concentrations of these were studied which include 0.25, 0.5, 0.75, 1, 1.25 and 1.5 (g or ml). For control a medium without the specific substance was used.

Effect of pH on lipase production

To study the effect of pH on enzyme production the pH of the basic medium was altered accordingly. The different pH used for the study include 4, 5, 6, 7, 8, 9 10, and 11.

Effect of agitation speed and inoculum size on lipase production

The five different agitation speed selected for the experiment was 30, 60, 90, 120, and, 150 rpm. One medium was maintained without any agitation, the control. The effect of inoculum size on lipase production was analyzed by inoculating the medium with five different quantity of pre-inoculum. The composition of the medium was same but the quantity of the medium was adjusted in such a way that the final volume is 100 ml after the addition of pre-inoculum. The different quantities of pre-inoculum used were 2.5, 5, 7.5, 10 and 12.5 ml.

Lipase assay

Lipase activity was estimated by the standard procedure [31]; paranitrophenyl palmitate (PNPP) was used as the substrate. The reaction mixture constituted 9 ml of 50 mM Tris HCl, pH- 8 comprising of 40 mg of triton X- 100 and 10 mg of gum arabic mixed with 3 mg of PNPP in 1 ml propane- 2-ol. Centrifuged cell free crude enzyme (0.1 ml) was added to 0.9 ml of reaction mixture, and incubated at 37°C for 30 minutes in a water bath. The reaction results in a yellow-coloured solution due to release of p- nitrophenol which was measured calorimetrically at 410 nm. The amount of enzyme required to release 1µmol of p-nitrophenol per minute per ml from PNPP was considered as the unit activity. P- nitrophenol was taken as standard.

RESULTS AND DISCUSSION

Culture medium and incubation time

Out of the four medium used the second medium was found to be the best for lipase production with a maximum lipolytic activity of 24.52 U/ml. The least amount of activity was observed for medium four 2.98 U/ml. Maximum activity of 13.96 U/ml was seen in medium one and 8.57 U/ml was observed for medium three respectively (Fig 1).

Effect of incubation time on lipase secretion was studied for a period of 120 h. Assay was done at every 24 h for lipase quantification in all four-medium studied. The second medium was found to be the best for inducing lipase production. The maximum lipase activity was observed after 24 h of incubation at normal room temperature. The activity was found to be 24.12 U/ml which was later found to decrease with time till 72 h and later increases at 96 h. The lowest activity recorded at 72 h was 12.35 U/ml. Maximum lipase productions for *S. marcescens* MBB05 occurred at 20 h [20]. Mohanasrinivasan and partners reported maximum activity at 48 h and lowest at 120 h [18], while Prasad observed best lipolytic activity after 45 h of incubation for a *S. marcescens* strain isolated from industrial effluents [20]. From the study it is clear that in the presence of olive oil the common substrate in all four medium lipase was induced. So, it can be inferred that it is not the substrate but other medium variables that is affecting lipase production.

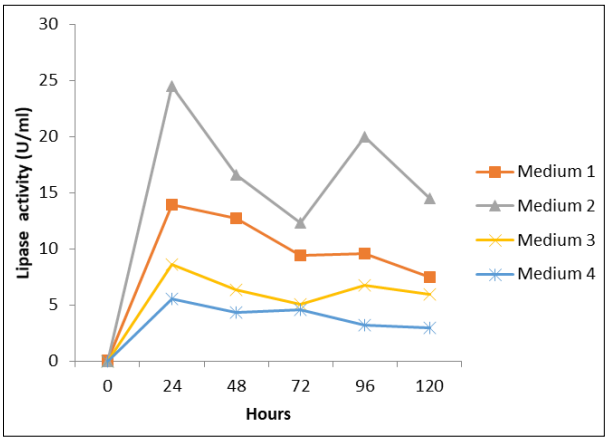

Fig 1 Lipase activity of SMVT 1 in different media

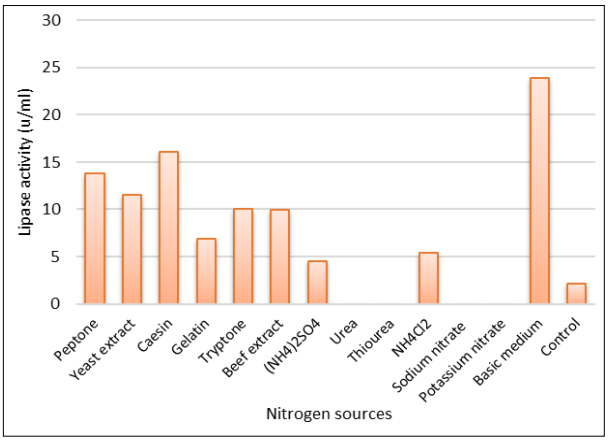

Fig 2 Effect of nitrogen sources on SMVT 1 lipase production

Nitrogen sources

Effect of nitrogen sources were studied by using only one nitrogen source at a time instead of two (yeast extract and peptone) in the basic medium. Organic nitrogen sources were found to be most promising for lipase. Maximum activity was found when casein was used as nitrogen source (16.08 U/ml), similar result was observed in the case of *Serratia rubidaea* [11], and extracellular lipase of *Serratia marcescens* [20]. Peptone and yeast extract two other organic nitrogen sources were found to be good for lipase production with an activity of 13.78 U/ml and 11.50 U/ml respectively (Fig 2). Organic nitrogen sources like peptone and yeast extract were reported to effectively induce high yield of lipase production in *Pseudomonas* and *Bacillus* [17], [25]. Other organic nitrogen sources also imparted good lipase activity, with least activity of 6.87 U/ml for gelatin. Among the nitrogen sources the inorganic ones like sodium nitrate, potassium nitrate, urea, and thiourea was found to be inhibitive as lipolytic activity was not recorded, in contrast to the control where lipase activity was observed. Ammonium salts was found to be the best inorganic nitrogen sources for lipase induction with a lipolytic activity in the range 4 – 5.4 U/ml. Influence of ammonium salts were previously reported [17]. When compared with the basic medium where a combination of two nitrogen sources, peptone and yeast extract was used in equal proportion, the activity was found to be more compared to the single nitrogen source casein and almost equal to the combined sum of the individual activities of peptone and yeast extract.

Carbon sources

Basic media contains carbon in the form of substrate only that is olive oil. In order to study the effect of carbon sources on lipase production specific carbon sources were added to the basic composition. Sucrose was identified as the best carbon source for lipase secretion (12.48 U/ml), followed by lactose and starch (Fig 3). Starch was the suitable carbon source followed by sucrose for *Serratia rubidaea* lipase [11], starch and glucose were the best source for *Serratia marcescens* [20]. In the presence of glucose

SMVT 1 recorded a lipolytic activity of 5.473 U/ml. Least amount of lipolysis was observed in the presence of complex carbon sources xylan (1.84 U/ml) and CMC (2.37 U/ml). When compared to the control basic medium the presence of an extra carbon source other than olive oil, the activity was found to decrease by 50-90%. Supplementation of basal lipase medium containing olive oil with carbon sources like glucose depressed the lipase production; this may be due to catabolic repression [17].

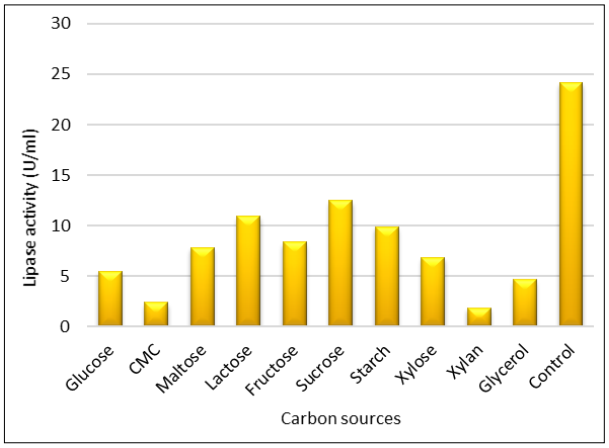

Fig 3 Effect of carbon sources on SMVT 1 lipase production

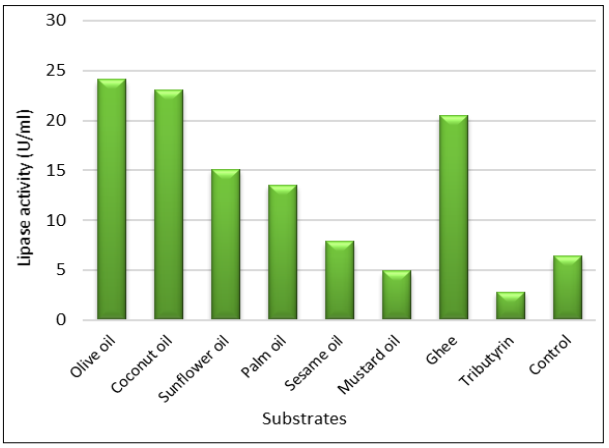

Fig 4 Effect of substrates on SMVT 1 lipase production

Substrates

Substrates used in the study were lipid carbon sources mainly natural oils and triglyceride like tributyrin. Carbon source in the form of lipids can effectively stimulate lipase synthesis [9, 13]. All the eight substrates induced varying level of lipase synthesis confirming lipid induced lipase synthesis by SMVT 1. Olive oil induced the maximum activity (24.11 U/ml); followed by coconut oil (23.07 U/ml) and ghee (20.49 U/ml). Tributyrin with 2.82 U/ml was the worst inducer (Fig 4). Similar result was reported by Mobarak- Qamsari et al, olive oil was found to be the best and tributyrin the worst inducer [17]. Olive oil was the best inducer for *S. marcescens* VITSD2 [18], and *Bacillus* sp [19], mustard oil and castor oil for *Pseudomonas* sp, [28 and 26]. Gingly oil maximized the lipase production in *S. marcescens* and *S. rubidaea* [20, 11]. The lipolytic activity for control was estimated to be 5.07 U/ml which is higher than tributyrin and mustard oil. *S. rubidaea* produced 17 U/ml of lipase activity in the absence of any substrate [12].

Effect of varying concentration of best nitrogen, carbon and substrate sources

From the initial studies it was clear that sucrose, casein and olive oil were the best carbon, nitrogen, and substrate that induced lipase production. Different concentration of sucrose, casein and olive oil on lipase secretion was analyzed separately. Maximum enzyme activity was observed when 1 g of casein, 0.5 g of sucrose, and 1 ml of olive oil was used in the medium (17.88 U/ml, 22.57 U/ml and 12.01 U/ml) respectively (Fig 5). Lipase production peaked under the absence of sucrose with olive oil as the sole carbon source in media. For casein and olive oil the lowermost activity was observed at the lowest concentration of 0.25 g and 0.25 ml, while for sucrose the lowest activity recorded was at highest concentration of 1.5 g (7.39 U/ml). Maximum lipase activity of *Serratia rubidaea* was registered when 24 g/l of casein, 4 g/l of starch, and 15ml/l of gingily oil was used [11]. Mohanasrinivasan and colleagues acquired maximum lipase induction with 0.7 ml of olive oil for *S. marcescens* VITSD2 [18]. One percentage palm oil (v/v) proved to be the best lipid carbon source on lipase production in marine actinomycete isolate, ABT – 206 [27]. Maximal lipase induction by *Aspergillus niger* in free and immobilized state was observed at 1% olive oil [4].

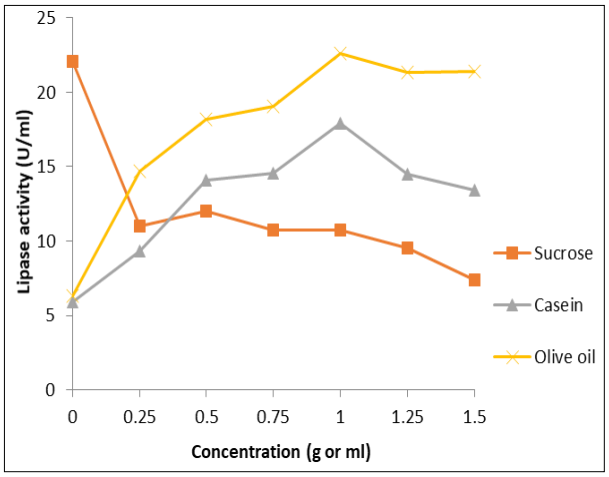

Fig 5 Effect of different concentrations of sucrose, casein, and olive oil on SMVT 1 lipase production

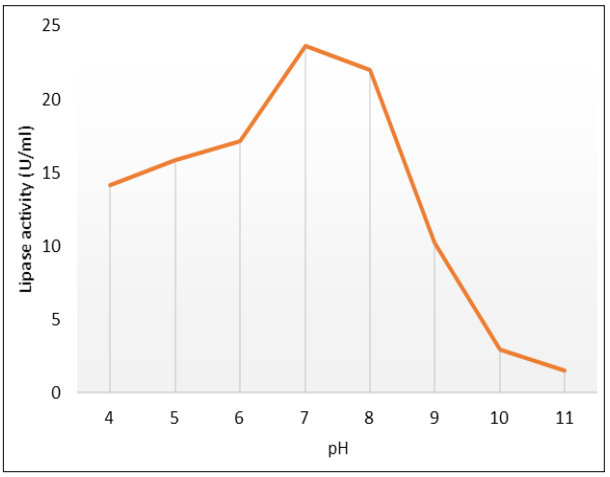

Fig 6 Effect of different pH on SMVT 1 lipase production

Effect of pH

Neutral pH and slightly basic pH was found to be most effective for inducing lipase secretion from SMVT 1. Maximum lipase production was observed at neutral pH, 23.63 U/ml and second highest at pH 8 (21.99 U/ml) (Fig 6). Gao et al, and Prasad from their study detected maximum lipase production by *Serratia marcescens* at pH 6.5, and 7 respectively [5 and 20]. Venil and coworkers reported pH 7 as optimum pH for lipase production by *Serratia marcescens* SB08 [29]. For *Serratia rubidaea* alkaline pH was most suited for lipase production while *Bacillus* sp preferred pH of 7.6 [19]. With increase in pH a drop in activity was observed, at pH 9 a steep fall in lipase activity by 13.38 U/ ml compared to pH 7. At pH 10 and 11 the activity dropped further with only 6% of activity being recorded at 11 compared to 7. At acidic pH the lipase induction was much higher compared to basic pH with an activity ranging from 14-17 U/ml.

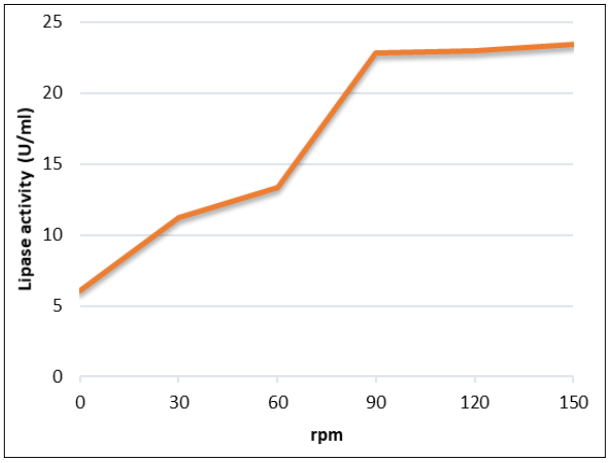

Fig 7 Effect of agitation speed on SMVT 1 lipase production

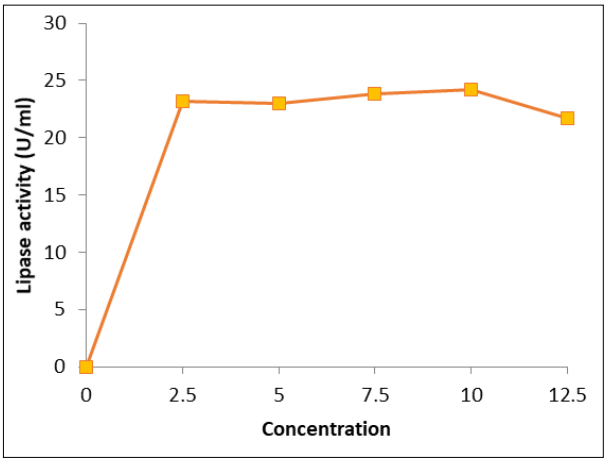

Fig 8 Effect of inoculum size on SMVT 1 lipase production

Effect of agitation speed and inoculum size

Agitation influences the growth of bacteria and the enzyme production by increasing the oxygen transfer and improving the dispersal of substrate and other media components allowing maximum contact with the organisms [6], [14]. Agitation speed ranging from 30-150 rpm with an interval of 30 rpm was studied for its effect on lipase induction. One sample was maintained without shaking as control. An enzyme activity of 6.03 U/ml was estimated for the control after 24 h of incubation. From 30-90 rpm increase in activity by 11 U/ml was observed. At 120 and 150 rpm an activity of 23 U/ml was recorded, only a small increase in activity by 0.4 U/ml was seen with upswing by 30 rpm. An overall increase by 25.77 % in lipase activity was observed with increase in rpm from 0-150 (Fig 7). This shows the effect of aeration and dependence on oxygen by SMVT 1 for lipase secretion. Long et al in the optimization of *Serratia marcescens* lipase explained the effect of agitation speed; increased shaking speed increases the dissolved oxygen concentration which in turn enhances the yield of lipase [15]. Salwoom *et al.* [23] obtained optimum lipase production at 150 rpm for *Pseudomonas* sp. LSK25.

The effect of inoculum size on lipase production was studied by inoculating medium two with five different volume of pre-inoculum. This gives a proper idea about the influence of cell number on lipase production and activity. Inoculum size was found to have least effect on lipase production after 24 h of incubation. Lipase activity within the range of 21-24 U/ml was observed for the different concentrations studied. Least activity was observed for highest concentration of 12.5 ml (21.72 U/ml). Highest lipolytic activity was seen when 10 ml of pre-inoculum was used (24. 19 U/ml) (Fig 8). Studies by Gao and team resulted in an inoculum size of 2.5 % (v/v) to be most effective in lipase production for *S. marcescens* ECU1010 [5]. Mohanasrinivasan and group reported maximum lipase production when an inoculum size of 4 % (v/v) 24 h seed culture was used [18]. With increase in inoculum size from 2.5 ml to 10 ml only a small increase by 5% was only observed while a decrease by around 9 % was seen with increase from 10 ml to 12.5 ml.

Prodigiosin is an important natural pigment produced by *Serratia marcescens*, *Vibrio psychroerythrus* and some other eubacteria. It is known to possess anticancer activity, immunosuppressive potential and dyeing properties [30]. Prodigiosin production in *S. marcescens* is influenced by environmental, nutritional and physiochemical factors [7]. During the experiment the effect of various carbon and nitrogen sources, and pH on prodigiosin production was noticed. Most of the carbon sources were found to be effective, glucose had maximum impact. Among the nitrogen sources yeast extract enhanced the prodigiosin production (Fig 9-10). In acidic, neutral and slight alkaline pH red colouration of prodigiosin was visible but in high alkaline pH prodigiosin appeared yellowish in colour.

CONCLUSION

Lipase with good activity and unique properties is of significant importance in major industries and eco-conservation; bioremediation and conversion of lipid wastes. Optimization studies are always necessary for maximizing enzyme production with minimum expense. The preliminary study showed sucrose, casein, olive oil, neutral pH, agitation speed of 120-150 rpm, and 24 h of incubation period to be most effective factors for extracellular lipase production from SMVT 1. Lipase activity of SMVT 1 was higher in the basal medium with olive oil as the solitary lipid carbon source and a mixture of complex nitrogen sources like peptone and yeast extract. Presence of a second carbon source in basal medium depressed the lipase generation, as higher activities were obtained in the presence of five lipid carbon substrates.

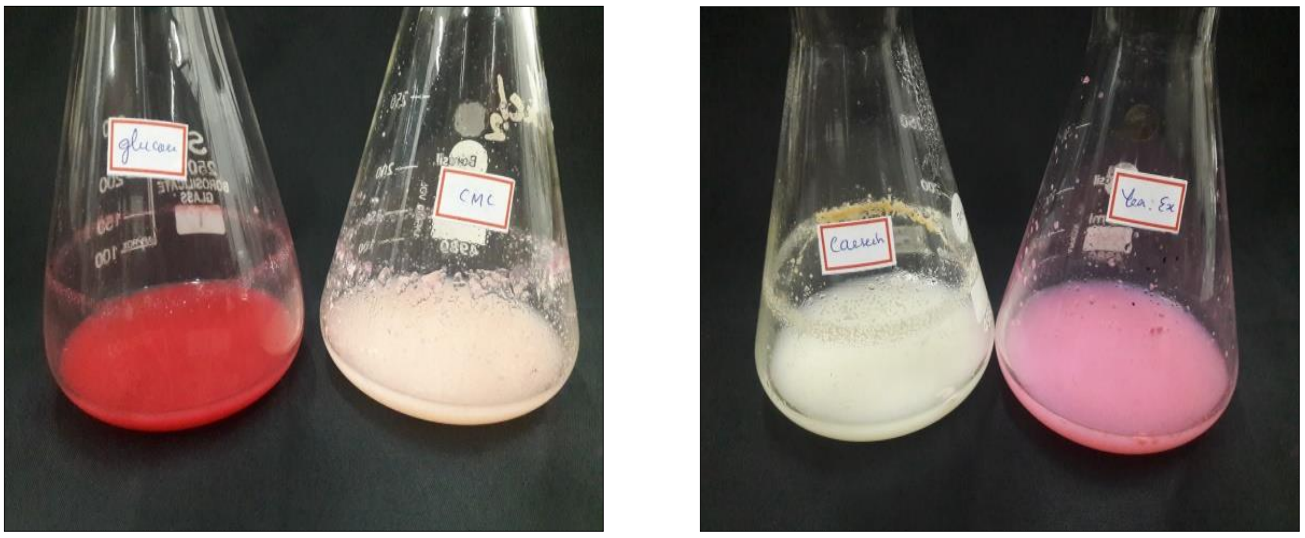

Fig 9-10 Prodigiosin production under glucose and yeast extract

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