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Screening, Production and Characterization of Industrially Important Enzymes by *Serratia marcescens* Strain VT1

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ABSTRACT

Microorganisms and their enzymes are important part of industrial biotechnology because of their high adaptability, versatile metabolic machinery and simple genetic constitution which can be easily manipulated to meet different needs. A mesophilic novel strain of *Serratia marcescens* - VT1 was found to produce extracellular protease (13.173 U/ml) and cold active and stable psychrophilic lipase (23.17 U/ml). Characterization of protease showed the enzyme to be active over a wide range of pH 4-11, optimum pH 10, and optimum temperature 50°C. SMVT1 lipase was found to be active from pH 7-9 with optimum pH 7 and good stability for 90 minutes, optimum temperature 30°C and to retain almost 80 % activity at lower temperatures (20 and 10°C). SMVT 1 protease was stable in hydrophilic solvents like methanol and ethanol. Lipase showed stability in both hydrophobic and hydrophilic solvents, hexane-92.67%, methanol-87.4% and acetone-85.23%. The wide range pH active protease and psychrophilic and organic solvent stable lipase can be made use in various sectors like bioremediation, waste management, and chemical synthesis, food processing and detergent additives.

KEY WORDS: *SERRATIA MARCESCENS*, PROTEASE, LIPASE, PSYCHROPHILIC, SOLVENT STABILITY.

INTRODUCTION

The importance of microbial enzyme lies in the role played by these bio-molecules in catalyzing the broad array of reactions which is indispensable for maintaining life in earth. Enzymes can be isolated from any living forms and each one has its own characteristic property which separates it from other. In industrial sector enzymes have immense applications, among these enzymes microbial ones form an inevitable part and have always been in demand due to their unique properties (Liu and Kokare 2017). There is an increase likelihood for finding enzymes with distinctive characters in areas ranging from dry deserts to extreme cold Antarctic regions. Microbial enzymes are known to be more stable and catalyse a wide range of activities when compared to their counterparts (Hasan et al., 2006). Microbial enzymes have applications in food and beverage, diary, leather, paper and pulp, pharmaceuticals, fertilizer and detergent industries. They are also used for degradation and sustainable management of waste materials and production of bio-fuels. Among these enzymes protease remains the dominant one till to date followed by cellulase and lipase (Chapman et al., 2018 Eddehech et al. 2019 Chandra et al 2020).

Proteases (EC 3.4.21) are enzymes that increases the rate of proteolysis resulting in single amino acids, the end product of protein breakdown resulting from the cleavage of peptide bond (Barrett and McDonald, 1986). Around 60 % of enzyme that is being marketed worldwide is protease (Rao et al., 2009). About two third of the commercial protease is of microbial origin (Beg and Gupta 2003).

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Lipases (EC 3.1.1.3) are hydrolytic enzymes that generate fattyacids and glycerol by fat digestion in aqueous media. Majority of industrial lipases are microbial ones, bacterial or fungal origin (Jaeger and Reetz 1998). Cellulases (EC 3.2.1.4) are a miscellany of endocellulases, exocellulases and cellobiose that generate cellulolysis the degradation of cellulose the most abundant carbon source in earth. Majority of cellulose in earth is considered as waste, but they can be the best source of food and potential source of energy (Elder et al., 1986). Xylanases (EC 3.2.1.8) are major hemicellulases that breaks down xylan a major plant cell wall material into xylose. Xylanases and cellulases are mainly of microbial origin and have also been reported from certain marine algae, snails, crustaceans, insects and seeds of plants (Walia et al., 2013 Chandra et al 2020).

Microbial protease, lipase, cellulase and xylanase are used in textile, pulp and paper, food and beverage and animal feed industries. They are also used in the production of biofuels, and eco-friendly bioconversion and management of waste materials and in pollution control (Liu and Kokare 2017, Subramaniyan and Prema 2002, Ramnath et al., 2017, Ali et al., 2016, Romdhane et al., 2010). Proteases and lipases have importance in medicinal and pharmaceutical sector (Andualema and Gessesse 2012, Singh et al., 2016). They are also used for degumming in silk and leather industry and for synthesis of peptides and esters. Proteases are made use in recovery of silver from photographic films and X-ray, (Ali et al., 2016). The ever increasing demand for microbial enzymes is because of the specific substrate specificity, low and high temperature and pH stability and optima (Singh et al., 2016, Treichel et al., 2010). For an enzyme to be industrially useful it must be thermo stable and must maintain activity even in the presence of solvents (Hasan et al., 2006). Enzymes which are fundamentally stable and active have more use in industrial sector. The following paper deals with the screening, production and characterisation of four industrially important enzymes, protease, lipase, cellulose and xylanase by *Serratia marcescens* strain VT 1.

MATERIAL AND METHODS

Microorganism is enough: The microorganism used in the present work was *Serratia marcescens* strain VT 1 (SMVT1) was isolated from oil contaminated soil collected from Althara Devi temple in Trivandrum district of Kerala. The organism was maintained by sub culturing in olive oil enriched nutrient agar plates (peptone - 0.5 g, yeast extract – 0.5 g, NaCl - 0.5 g, agar - 2 g & olive oil 1 ml for 100 ml). The cultures were incubated at 37°C for two days and were stored at 4°C in refrigerator.

Screening for enzymes – plate assay: Protease production:Casein agar plates composed of casein - 2 g, agar- 2 g, glucose - 0.5 g, NaCl - 0.5 g, peptone - 0.5 g & yeast extract - 0.5 g in 100 ml distilled water were streaked with microbial culture and kept in incubator for 2 days at 37°C. The plates were observed for clear zones around colonies which confirm protease activity.

Lipase production: Rhodamine olive oil agar plates were used to screen the presence of extracellular lipase (Castro-Ochoa et al., 2005). The media composed of peptone - 0.5 g, yeast extract - 0.5 g, NaCl - 0.5 g, agar - 2 g, olive oil - 3 ml & rhodamine - 0.1 mg for 100ml.The inoculated plates were incubated at 37°C for 48 h and observed under UV light for fluorescent orange halos around colonies which confirms lipase production.

Cellulase production: Screening test for cellulase was done by streaking the microbial culture on to CMC (Carboxy methyl cellulose) agar plates (CMC - 0.5 g, peptone - 0.5 g, yeast extract - 0.5 g & agar - 2 g for 100 ml). The plates were incubated for 48 h at 37°C. The plates were flooded with 1 M, 0.1% Congo red for 15 minutes and washed with 1M NaCl solution. Presence of clear zone confirms cellulase activity (Gohel et al., 2014).

Xylanase production: The bacterial culture was grown on xylan containing medium (peptone - 0.06g, yeast extract - 0.06 g, MgSO4 - 0.02 g, K_2HPO_4 - 0.1 g, xylan - 0.5 g & agar - 2 g for 100 ml) for 48 h at 37°C. Congo red (1M, 0.1 %) was used for staining and 1 M NaCl was used for destaining (Subramaniyan, 2012).Clear zone around the colony confirms presence of extracellular xylanase.

Fermentation studies: Medium for protease production:The pre- inoculums was raised at room temperature for 24 h at 120 rpm in 50 ml liquid medium composed of casein - 0.5 g, glucose - 0.5 g, NaCl - 0.5 g, peptone - 0.5 g & yeast extract - 0.5 g, $CaCl_2.H_2O$ - 0.05 g, KH_2PO_4 - 0.02 g & $MgSO_4.7H_2O$ - 0.05 g. 5 ml of this culture was transferred to production media of same composition and incubated at room temperature at 120 rpm for 96 h. pH of the medium was adjusted to 7 using Na_2CO_3 and the total volume of the medium was made up to 100 ml. Samples were collected at intervals of 24 h and centrifuged at 10,000 rpm for 15 minutes at 4°C, the supernatant was stored at 4°C in refrigerator and used as crude enzyme.

Medium for lipase production: The pre- inoculums was grown in 50 ml nutrient broth (yeast extract- 0.5 %, peptone- 0.5 % & NaCl- 0.5%) by transferring a loop full of one day old microbial culture, incubated at room temperature at 120 rpm for 24 h. The production medium contains 1% olive oil in addition to pre- inoculum media, pH- 7 (Selvamohan et al., 2012). The culture conditions were exactly same. Samples were collected at a regular interval of 24 h for 4 days. Crude enzyme was obtained by centrifuging the culture to separate the cells at 10,000 rpm for 15 minutes at 4°C. The crude enzyme was stored at 4°C in refrigerator.

Protein estimation and pH determination: Cell protein was estimated according to standard procedure (Lowry et al., 1951). Bovine Serum Albumin (BSA) was used as standard. The pH values of the culture medium at different intervals were also noted.

Quantification of enzyme activity

Lipase assay: Lipolytic activity was determined by using paranitrophenyl palmitate (PNPP) as substrate (Yagiz et al., 2007). Reaction mixture (9 ml of 50 Mm Tris HCl pH - 8 containing 40 mg triton X- 100 &10 mg gum arabic mixed with 3 mg of PNPP in 1 ml propane- 2- ol) with 0.1 ml of crude enzyme was incubated at 37°C for 30 minutes, and the release of p- nitrophenol was measured calorimetrically at 410 nm. The amount of enzyme required to release 1µmol of p- nitrophenol per minute per ml from PNPP was defined as the unit enzyme activity. P- nitrophenol was used as the standard.

Protease assay: Protease activity was measured using casein as substrate (Tsuchida et al., 1986). 0.5 ml of crude enzyme was mixed with0.5 ml of substrate (2% casein in sodium phosphate buffer pH- 7) and incubated at 40°C for 10 minutes. The reaction was terminated by adding 1 ml of 10% TCA. 0.5 ml of phosphate buffer with substrate and 1 ml TCA was used as blank. The resulting mixture was centrifuged at 2000 rpm for 5 minutes. To 1 ml of supernatant 5 ml of 0.44 M Na_2CO_3 was added and incubated for 10 minutes. To this 2 fold diluted 0.5 ml Folin- Ciocalteau reagent was added and allowed to stand for 20 minutes; absorbance was measured at 660 nm. One unit of enzyme activity is defined as the amount of enzyme that released 1µmol of tyrosine per minute per ml. Bovine Serum Albumin (BSA) was used as standard.

Biochemical characterization: The biochemical characterization of crude enzyme was carried out with respect to optimum pH, pH stability, optimum temperature, temperature stability and effect of solvents.

Optimum pH and pH stability: Optimum pH for enzyme activity was determined at different pH values ranging from 4-11 by preparing substrates in appropriate buffers (protease: 4 & 5- sodium acetate buffer, 6 to 8- sodium phosphate buffer and 9 to 11- sodium carbonate buffer, lipase: 4 & 5- sodium acetate buffer, 6- sodium phosphate buffer, 7 to 9- Tris HCl and 10 & 11- sodium carbonate buffer). Enzyme assays were done according to the above mentioned procedures. The effect of pH on enzyme stability was studied by incubating the enzyme in desired buffer with optimum pH for two hours and calculating the residual activity for every half an hour.

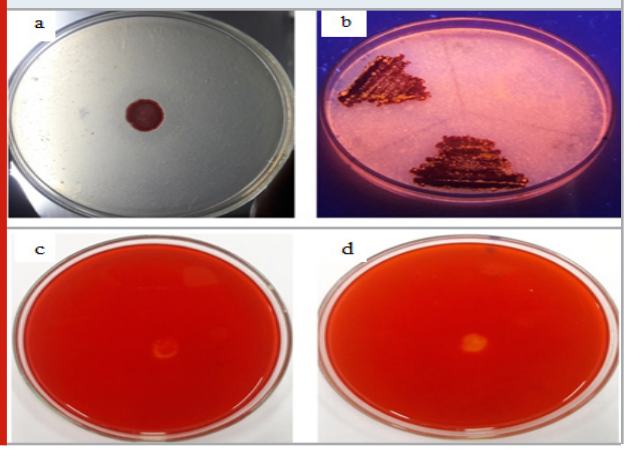
Optimum temperature and temperature stability: The optimum temperature was determined by performing respective enzyme assay at different temperatures ranging from 30- 80°C. Thermal stability was estimated by pre-incubating the crude enzyme at optimum temperature for two hours. Residual activity was determined for every half an hour.

Effect of solvents on enzyme activity: The different solvents used in the study ethanol, methanol, butanol, isopropanol, chloroform, ethyl acetate, acetone and hexane were selected based on their log P values. Crude enzymes were mixed with solvents to make a final concentration of 20% (v/v); the mixture was mixed well and stored at 4°C. The residual activity was determined after two hours of incubation by colorimetric method. Enzymes mixed with distilled water 20% (v/v) was used as control.

RESULTS AND DISCUSSION

S. marcescens VT 1 was subjected to screening by plate assays to confirm the presence of extracellular proteases, lipases, cellulases and xylanases (Fig: 1). After 48 h of incubation clear zone was visible around the bacterial colony in casein agar plates confirming protease activity. Hydrolysis of casein and gelatin was used as the preliminary test for isolation of protease producing *Serratia* sp RSPB11 (Bhargavi and Prakasham 2012). 14 protease producing strains were isolated on casein agar plates (Vakilwala and Patel 2017). Under UV light orange fluorescent halos were clearly visible resulting from the reaction of fatty acids and rhodamine pointing towards expression of extracellular lipase. Extracellular lipase production of *Serratia marcescens* and *S. aureus*, on Rhodamine B plates have been previously reported (Kouker and Jaeger 1987). Ejike and coworkers (2017) used Rhodamine B plates for preliminary screening of lipase producing organism *S. marcescens*. Majority of the lipases produced by microorganisms are extracellular inducible enzymes transmitted to outer surface (Ota et al., 1982). Clear zones were completely absent in CMC agar plates and xylan agar plates, so it can be inferred that cellulase and xylanase production is absent, therefore as the next part of work the production pattern and biochemical characterization of protease and lipase were studied.

Figure 1: Plate Assay (A) Protease, (B) Lipase, (C) Cellulase And (D) Xylanase From SMVT1



The enzyme production pattern was evaluated along with the change in pH profile of the medium and concentration of cell protein for a period of 96 h (Fig: 4 and 5). The extracellular protease production started with the log phase of growth and activity reached the peak value after 24 h (13.173 U/ml), thereafter the activity declined with time and showed a slight increase at 96 h (Fig: 2). The lipolytic activity of *S. marcescens* VT 1 was measured by colorimetry using PNPP as substrate at 420

nm. Study showed that the lipase activity was expressed maximum after 24 h of incubation, further a turn down in activity is seen which is followed by a rise in activity at 96 h (Fig: 3). The maximum activity recorded was 23.17 U/ml followed by 20.16 U/ml.

Figure 2: Enzyme Activity- SMVT1 Protease

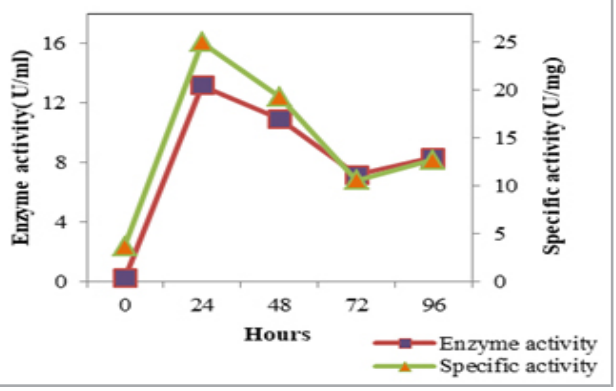
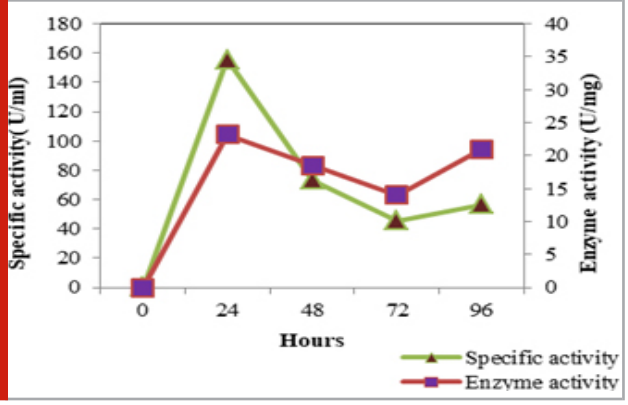


Figure 3: Enzyme Activity- SMVT1 Lipase



S. marcescens protease has been studied and described in previous papers (Henriette et al., 1993, Romero et. al., 2001, Ustariz et. al., 2008). Excretion of extracellular protease of *S. marcescens* ATCC 25419 was reported to occur during the logarithmic growth phase and was determined to be highest during the stationary growth phase et al., (Schmitz and Braun 1980). Lipid hydrolysis by *S. marcescens* was estimated and recorded earlier (Heller 1979, Prasad 2013, Abdou 2003). Henriette et al., (1993) based on their study reported that the *S. marcescens* released lipase during the stationary phase of growth after growing exponentially at 22°C for 6 h. Lipase production was found to begin after 5- 10 hours of growth at 27°C (Makhzoum et al., 1995). Proteolytic activity along with lipolytic activity of *S. marcescens* has been previously studied and recorded (Henriette et al., 1993, Begam et al., 2012, Abdou and Ohashi 1996). The rise in activity at 96 h may be due to the release of intracellular lipase into the medium due to death and lyses of bacterial cells. The pH of the protease medium was found to shift towards the alkaline side with time and growth of *S. marcescens* VT 1, while the pH of lipase medium turned alkaline first and later the alkalinity started to drop off after 48 h. The release of fatty acids, the byproduct of lipolysis into the medium could have resulted in reduction of alkalinity.

Figure 4: Cell Protein Profile Variation During Protease And Lipase Production By SMVT1.

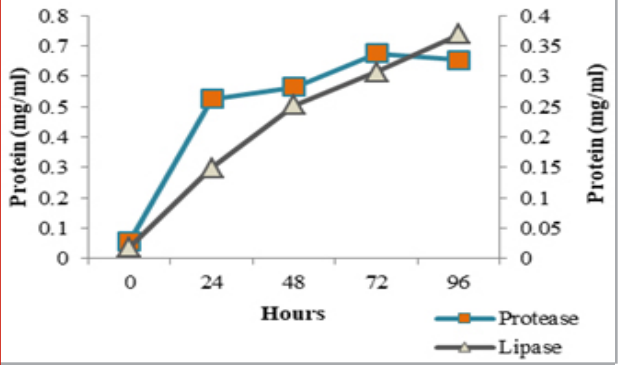


Figure 5: Variation In pH Values During SMVT1 Protease And Lipase Production.

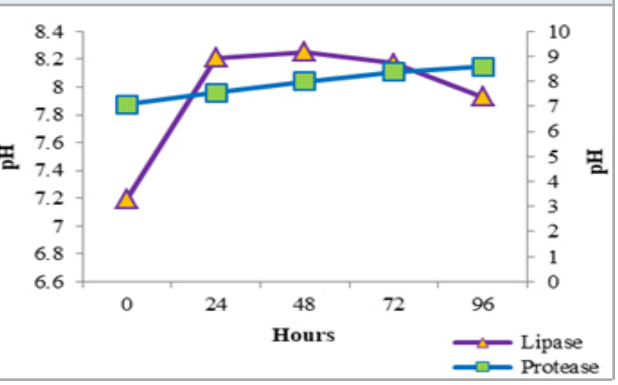


Figure 6: Optimum pH Of Protease And Lipase

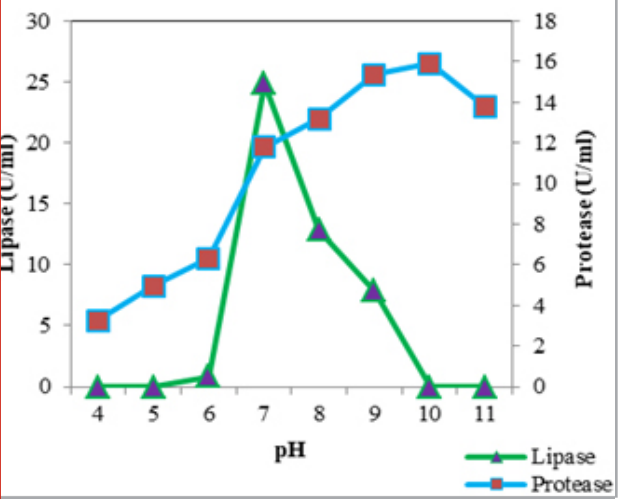
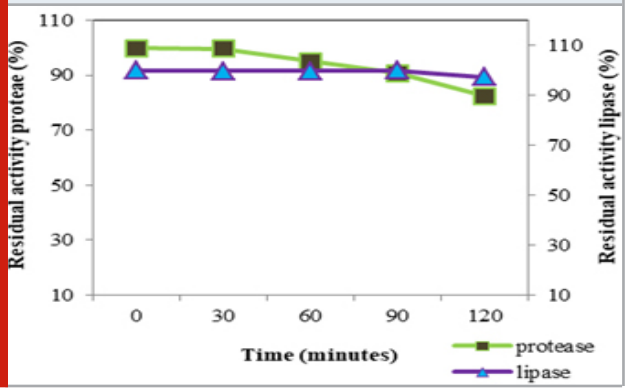
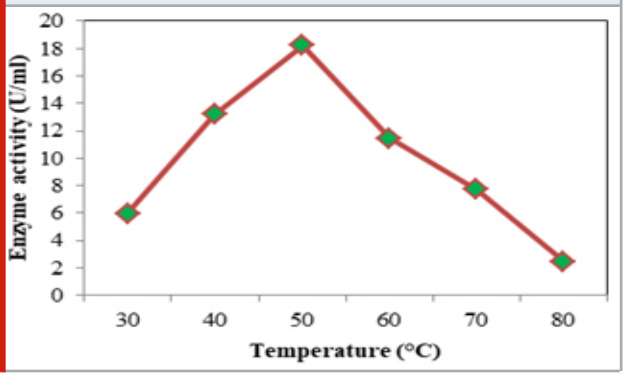


Figure 7: Ph Stability o Protease and Lipase



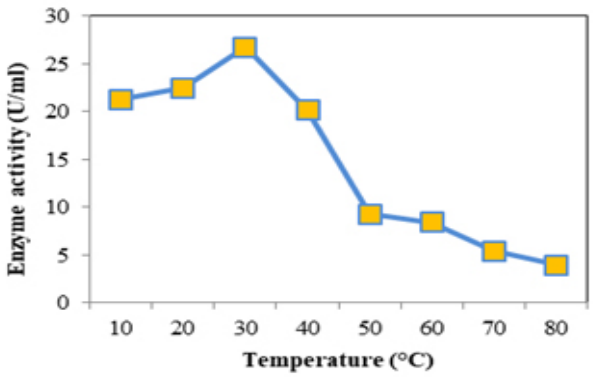
The optimum pH (Fig: 6) for protease was found to be 10 (15.887 U/ml). At pH 9 the activity was found to be almost similar (15.346 U/ml). The proteolytic activity was found to decrease as the pH shifted towards acidic range. From this it is clear that the lipase of *S. marcescens* VT 1 is an alkaline protease and is active over a wide range of pH. Annapurna and colleagues (Annapurna et al., 2012) reported an alkaline protease form *S. marcescens* with optimum pH 10. *S. marcescens* metalloprotease SMP 6.1 with pH optimum of 10 was also reported (Salamone and Wodzinski 1997). Protease with an optimum pH of 8 and pH range of 6 to 11 was recorded in *S. marcescens* (Femi-Ola et al., 2014). Kim et al., (2007) reported the optimal pH of protease by *S. marcescens* as 7 using casein substrate. *S. marcescens* TKU019 protease exhibited a broad pH range 5-10 (Liang et al., 2010). The optimum activity of lipase was estimated at neutral pH. A sharp decline in activity was observed when pH shifted to 6 and activity was found to be absent at pH 5 and 4. Activity was found to decrease as the pH was raised and was lost at pH 10 and 11. *S. marcescens* lipase optimum pH was found to be 6.5 (Gao et al., 2004), 8 and retained 95% of maximum activity at 7 (Zaki and Saeed 2012) and 8-9 (Abdou 2003).

Figure 8: Optimum Temperature Of SMVT1 Protease



The pH stability (Fig: 7) of VT 1 protease was studied by incubating the crude enzyme in pH 10 for two hour at 4°C; assay was conducted every half an hour. The enzyme was found to be stable for 30 minutes in pH 10, after 60 minutes the enzyme retained 96.15% activity. After 2 h the enzyme lost 17.63% of its original activity. *S. marcescens* protease was found to be stable over a pH range of 5 to 10 under low temperature incubation and underwent inactivation in alkaline pH on incubation under elevated temperature (Miyata et al., 1970). Purified *S. marcescens* protease remained stable for 1 h at pH ranging from 6-9 and lost 40% of stability at pH 10 (Iqbal et al., 2018). For lipase pH stability study was conducted by incubating enzyme in Tris HCl buffer pH 7 and estimating activity every 30 minutes for two hours. VT 1 extracellular lipase was found to be stable for one and half hours and lost 2.73% of the original activity in the next 30 minutes and retained 97.27% activity by the end of two hours. *S. marcescens* lipase showed good stability between pH 6-9 (Makhzoum et al., 1995).

Figure 9: Optimum Temperature of SMVT1 Lipase



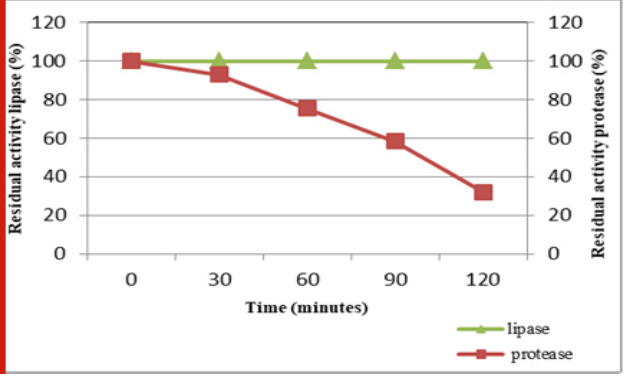
Lipase from *S. grimisii* remained stable over the pH range 7-9 et al., (Abdou 2003). Abdou et al.,(2003) found that purified *S. marcescens* lipase showed maximum stability at pH 8. In a study by Zaki and Saeed (2012) purified lipase from *S. marcescens* showed maximum stability at pH 8.

Optimum temperature for protease was assayed at temperature from 30 to 80°C; casein was used as the substrate at pH 7. Optimum activity recorded was 18.232 U/ml seen at 50°C (Fig: 8). At higher and lower temperatures the activity was found to decrease. For alkaline proteases the optimum temperature falls in the range 50- 70°C (Kumar and Takagi 1999). Femi-Ola and fellow workers (2014) obtained optimum proteolytic activity for *S. marcescens* at 50°C, at temperatures above and below reduction in activity was observed. Optimum activity was reported at 67°C for *S. marcescens*, alkaline protease (Annapurna et al., 2012). The optimum temperature of *S. marcescens* protease was found to be 50°C (Liang et al., 2010) and 42°C (Salamone and Wodzinski 1997). Optimum temperature for lipase (Fig: 9) was initially evaluated at temperature range 30- 80°C. Since the optimum activity was at 30°C (26.736 U/ml), lower temperatures 10 & 20°C were also considered. Optimum temperature for lipase from *S. marcescens* was determined as 35°C (Zaki and Saeed 2012).

S. marcescens lipase optimum temperature was reported to be 37°C (Abdou 2003), between 25- 35°C (Immanuel et al., 2008). With increase in temperature the lipolytic activity was found to decrease showing a sharp decline from 40- 50°C. At lower temperatures (30 to 10°C) the enzyme activity showed a slight decrease only, the activity was found to decrease by only 5.4 U/ml retaining almost 80% of the maximum activity. This shows the psychrophilic property of lipase. Abdou (2003) observed high lipase activity for *S. marcescens* at 5°C and low temperature activity of *S. grimisii*. Cold active enzymes have much economic and ecological benefit when compared with their equivalents which need high temperature to function (Marchi et al., 2007) Psychrophilic lipases have high catalytic activity and consume less energy at low temperatures which makes them efficient tools in the production of detergent, leather, food, pharmaceuticals, fine chemical and bioremediation (Joseph et al., 2008).

Temperature stability of VT 1 protease and lipase (Fig: 11) were studied at 50°C and 30°C (optimum temperature) respectively for a period of 2h and residual activity was determined after every half an hour. The VT 1 protease was active throughout the study period of two hours, but the activity was found to decrease drastically after 30 minutes. After 30 minutes of incubation at 50°C the activity decreased by 7%, thereafter the activity was found to lower by 61%. The lipase of *S. marcescens* was stable up to 45°C and lost 75% of stability at 60°C after 1 h of incubation (Iqbalet al., 2018). Matsumoto and partners (Matsumoto et al., 1984) showed the complete inactivation of *S. marcescens* protease above 65°C. Miyata et al., (1970) displayed the protease fro *Serratia* spp. to be relatively stable at 40°C throughout the 60 min of study, but mere heating for 15 min at 50°C resulted in complete inactivation.

Figure 10: Temperature Stability of SMVT1 Protease And Lipase.



The lipase of VT 1 was thermo stable throughout two hours, showing that the lipase is highly stable at lower temperature. The *S. marcescens* lipase showed least residual activity at 85°C and was completely inactivated at 90°C. *S. marcescens* lipase was exhibited to be less thermostable compared to other psychrotroph lipases, at the same time is resistant to inactivation at lower temperatures (Abdou 2003). Thermal stability of *S. marcescens* lipase in the culture was showed to decrease with increasing temperatures (Gao et.al., 2004. After 24 h the residual activity was found to decrease by 61% and 36% after incubation at 35°C and 25°C.

Table 1. Solvent stability of SMVT1 protease and lipase

Solvents	log P	Residual protease activity (%)	Residual activity (%) lipase
Methanol	-0.69	79.71	87.4
Acetone	-0.61	54.73	85.23
Ethanol	-0.18	82.28	76.55
Isopropanol	0.16	20.06	28.09
Ethyl acetate	0.71	38.83	37.63
Butanol	0.839	59.34	34.73
Chloroform	1.67	33.83	71.39
Hexane	3.769	65.79	92.67

Solvent stability of the enzymes were studied in eight different solvents with different log P values, which represents hydrophobicity level (Table: 1). Lower the log P value lower the hydrophobicity. Protease was found to be stable in polar solvents like methanol and ethanol, unlike the previously reported solvent tolerant protease from *S. marcescens* MH6 which displayed notable stability in hydrophobic solvents (Wan et al., 2010). Protease showed some stability in nonpolar solvent hexane also. Least amount of residual activity was observed in isopropanol and chloroform. *Serratia* sp. SYBC H protease retained over 90% activity even after 60 minutes of incubation at 40°C in 50% (v/v) hydrophilic organic solvents such as dimethylformamide, dimethylsulfoxide, and acetone (Li et al., 2010). The *S. marcescens* PPB-26 protease showed maximum stability in methanol and ethanol and was stable in all organic solvents except isopropanol (Thakur et al., 2016).

The VT 1 lipase showed higher stability in methanol, acetone, and ethanol which are polar solvents with lower log P values, however maximum stability was observed in a nonpolar solvent hexane retaining 92% of activity after 2 h of incubation. Lipases displaying stability in a wide group of organic solvents, regardless of their log P values have industrial importance in esterification, interesterification, and transesterification alias synthesis (Chakravorty et al., 2012). *Serratia marcescens* ECU1010 lipase presented commendable stability in many water miscible and immiscible solvents (Zhaoet al., 2008). Lipase from *Pseudomonas reinekei* displayed significant stability in hydrophilic solvents like ethanol and methanol (20% v/v) after 24 h of incubation (Priyanka et al., 2019). The activation of lipase in hydrophilic and hydrophobic solvents can be explained due to the amino acid solvent interactions leading to opening of the lid or flap covering the catalytic site keeping the enzyme

in an open conformation (Bose and Keharia 2013, Cao et al., 2012).

CONCLUSION

S. marcescens strain VT 1 a soil bacterium was isolated for the production of industrially important enzymes and was found to produce lipase and protease. A further characterization study showed the protease to have an optimum pH of 10 and was active over a wide pH range of 4-11 and showed stability at pH 10. Lipase had an optimum pH of 7 and was active in a pH range of 7-9 with good stability at pH 7 for 90 minutes. The optimum temperature of protease and lipase was found to be 50 and 30°C. Lipase was found to be cold active and incredibly stable at low temperatures. SMVT 1 protease was found to be stable in hydrophilic solvents like ethanol and methanol, while lipase showed stability in solvents with both low and high log P values. *S. marcescens* strain VT 1 protease and lipase can be of great importance in industries like food processing, biodiesel production, and waste management.

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