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Characterisation of *Streptomyces violascens* OC125-8 lipase for oily wastewater treatment

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Abstract

In this study, the lipase-producing bacterium *Streptomyces violascens* (GenBank number MF621564) was identified, and the extracellular *S. violascens* OC125-8 lipase produced by this strain was characterised for use in wastewater treatment. The lipase was partially purified by ammonium sulphate precipitation at a final yield of 3.28-fold purification and a recovery of 56%. The *S. violascens* OC125-8 lipase exhibited optimum catalytic activity at 40 °C and pH 8.0; it was stable at 30–40 °C with more than 86% residual activity after 1 h; it was also stable over a relatively broad pH range of pH 7.0–11.0, retaining 83.3–100% activity. V_{max} and K_m values were calculated as 0.61 μmol/min/mg and 0.259 mM, respectively. Enzyme activity significantly increased in the presence of Fe^{2+} ion but was inhibited by Ca^{2+}, Mn^{2+}, Cu^{2+} and Mg^{2+}. The addition of a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), strongly inhibited enzyme activity while ethylenediaminetetraacetic acid (EDTA), a metal chelating agent, had no inhibitory effect. The enzyme was fairly stable in the presence of surfactants as well as sodium perborate. Examination of commercial detergent tolerance revealed that the lipase was strongly stable in Tursil (88%), Pril (97%) and Fairy (98.5%), while the lipase was activated in Omo (113.4%) and Ariel (128.3%). Moreover, the lipase showed highest activity towards olive oil (100%), sunflower oil (90%) and burned sunflower oil (55%), while corn oil (44%) and burned olive oil (15%) were less hydrolysed by the enzyme. These properties demonstrate that *S. violascens* OC125-8 lipase is an ideal choice for oily wastewater management.

Keywords *Streptomyces violascens* · Lipase · Characterisation · Wastewater · Oil degradation

Introduction

Wastewater from households, restaurants, dairy industries and food-processing facilities contain high concentrations of fats and oils which are the major contaminants in wastewater treatment systems. These substances form a thin film on the water's surface which prevents the exchange of oxygen into the water, consequently causing a reduction in oxygen saturation and retardation of biological degradation (Rosa et al. 2009). In addition, oily wastes solidify in cold temperatures, resulting in the clogging of pipes and drains which can lead to foaming and undesirable odours (Valladão et al. 2007). There are conventional physicochemical techniques for the removal of oils and fats, their main disadvantage being low efficiency rates (Abd El-Gawad 2014). To improve the efficiency of these traditional approaches, they can be combined with bio-degradation (Nzila et al. 2017).

The use of microorganisms or their enzymes in wastewater treatment has attracted attention because of the method's clean application and capacity to overcome existing limitations (Leal et al. 2006). In general, enzymes are highly efficient and specific catalysts (Nelson and Cox 2004) which can target the contaminant without affecting other components of the wastewater. An important advantage is that they can work under mild reaction conditions relating to temperature and pH (Mugdha and Usha 2012). They are also

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environmentally friendly, cost effective and do not require purification, thus allowing the treatment of huge quantities of wastewater (Rigo et al. 2008; Zhang et al. 2012).

Lipases play an important role in the degradation of waste oil and fat. The catalytic reaction in which they are involved occurs between the oil and the aqueous phases, and they have proved to be excellent at degrading and transforming complex long-chain and water-insoluble triglycerides into simpler free fatty acids (Jamie et al. 2016). The application of lipase-producing microorganisms or their enzymes to degrade oil and fat in wastewater has become an interesting strategy (Bhumibhamon et al. 2002).

Streptomyces are among the most highly studied and best identified bacteria due to their important role in ecology, medicine and the biotechnology industry (Bentley et al. 2002). These bacteria are generally non-pathogenic soil saprophytes (Clark et al. 2013). According to the genomic data available for *Streptomyces*, these bacteria have approximately 50–80 genes encoding putative lipolytic enzymes (Bielen et al. 2009). Nevertheless, only a few reports have focused on their enzymatic potential (Mander et al. 2012; Habbeche et al. 2014; Ugur et al. 2014; Ben Elhoul et al. 2015), and the characterisation and usability of their lipases for wastewater management have not yet been reported.

Various studies about the isolation, purification and characterisation of *Streptomyces* lipases exist (Mander et al. 2012; Ugur et al. 2014; Ayaz et al. 2015; Ben Elhoul et al. 2015); however, and to the best of our knowledge, there are no reports on their potential for use in wastewater management systems (Rajanikanth and Damodharam 2017). The present work reports for the first time the characterisation and potential use of lipase *S. violascens* OC 125-8 in wastewater treatment.

Methods

Microorganism and lipase activity

The lipase-producing isolate of *S. violascens* OC125-8 was obtained from Muğla Sıtkı Koçman University's Culture Collection. The lipase production of this bacterium has been previously detected on tributyrin and rhodamine-B agar plates, and spectrophotometrically using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate (Ugur et al. 2014).

The isolate was identified by amplifying 16S rRNA using the universal bacterial primers F(5′-AGAGTTTGATCCTGG CTCAG-3′) and R(5′-TACCAGGGGTATCTAATCCTGTT-3′). The conditions for thermal cycling were as follows: pre-denaturation at 94 °C for 30 s; denaturation at 94 °C for 30 s in each polymerase chain reaction (PCR) cycle; annealing at 53 °C for 30 s in each cycle; renaturation at 72 °C for 40 s in each cycle; and final elongation at 72 °C for 10 min after all 30 cycles were over. Following PCR, the amplicons were sequenced by RefGen (ODTU, Teknokent, Turkey), and the gene sequence was checked against those in the National Center for Biotechnology Information nucleotide sequence databases using BLAST.

The effect of development time on lipase production by *S. violascens* OC125-8 was studied in 250 mL of sterile International *Streptomyces* Project-2 (ISP$_2$) broth in a 500-mL Erlenmeyer flask. The ISP$_2$ broth was inoculated with 2% (v/v) *S. violascens* OC125-8 spore solution and incubated in a shaker at 30 °C for 168 h. Supernatant samples were taken periodically and lipase activity was determined spectrophotometrically using *p*-NPP (Boran and Ugur 2010).

Partially purification of the lipase

For partial purification of the lipase, 96-h-old culture was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was precipitated with ammonium sulphate in saturation of 80%. This suspension was then centrifuged in the above conditions. The precipitates were resuspended in a minimal amount of Tris–HCl buffer (pH 8.0). The protein content (Bradford 1976) and lipase activity in both the supernatant and the precipitate were determined.

Characterisation of the lipase

To determine the effect of temperature and pH on substrate degradation in the lipase, the lipase–substrate mixture was incubated at different temperatures (30–60 °C) and pH values. The different buffers used were citrate phosphate at pH 6.0, Tris–HCl at pH 7.0–9.0 and glycine-NaOH at pH 10.0–11.0. Due to the poor pH stability of *p*-NPP above pH 9.0, activity was determined using a titrimetric method with olive oil as a substrate at pH 9.0–11.0. The optimum temperature (40 °C) and pH (8.0) were identified and used in the present study.

To determine temperature and pH stability, the *S. violascens* OC125-8 lipase was preincubated using the above-mentioned reaction conditions for 1 h. Residual lipase activity was detected spectrophotometrically and initial lipase activity was regarded as 100%.

The Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}) values of *S. violascens* OC125-8 were detected by measuring the reaction velocities at the different concentrations of *p*-NPP (0.1–3.5 mM). The Lineweaver–Burk equation graphic was used to determine the kinetic parameters of the enzyme.

A standard spectrophotometric method was used to determine the effects on substrate degradation of the metal ions Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+}; of the boron compounds

sodium metaborate ($NaBO_2$), boric acid (H_3BO_3), potassium metaborate (BKO_2) and borax ($Na_2B_4O_7$); and of the enzyme inhibitors EDTA, sodium dodecyl sulphate (SDS) and PMSF. The enzyme was preincubated with these agents for 1 h at room temperature, and residual lipase activities were tested in optimum conditions. The enzyme solution without the addition of metal ions, boron compounds or enzyme inhibitors was used as a control.

The effect of oxidising agents (sodium hypochlorite, hydrogen peroxide (H_2O_2) and sodium perborate) and surfactants (Tween-40, Tween-60, Tween-80 and Triton X-100) on enzyme stability were detected by preincubating the lipase at room temperature for 1 h with these agents. Later, enzyme activity was measured and residual activities were calculated. Controls without oxidising agents or surfactants were used.

The stability of the enzyme in commercial detergents was determined using Ariel (Procter and Gamble, Belgium), Omo (Unilever, Turkey), Tursil (Henkel, Austria), Fairy (Procter and Gamble, Belgium) and Pril (Henkel, Austria). Diluted detergent solutions were prepared with tap water and heated at 100 °C for 1 h to inactivate the endogenous enzymes found in these detergents. The *S. violascens* OC125-8 lipase was preincubated with the detergent solutions for 1 h at room temperature and the lipase activities were tested using a standard spectrophotometric method. A detergent-free enzyme solution was used as a control.

Oil hydrolysis of *S. violascens* OC125-8 lipase was determined titrimetrically at optimum pH and optimum temperature conditions using olive oil, corn oil, sunflower oil, burned olive oil and burned sunflower oil according to Ugur and Boran (2014).

All experiments were repeated three times. The results are presented as mean and standard deviation.

Results and discussion

Identification of bacteria and lipase activity

The 16S rRNA gene sequencing results analysed with Gen-Bank identified isolate OC125-8 as *S. violascens* (GenBank accession number MF621564).

Initial lipase activity of *S. violascens* OC125-8 was observed after 48 h of incubation and reached its maximal value at 96 h of incubation. After 120 h, activity began to decrease (data not shown). The decrease in activity might have been due to deactivation of the enzyme by proteases or some toxic secondary metabolites released during growth. In other studies, the highest *Streptomyces* lipase activity was typically obtained after 120 h (Ugur et al. 2014; Ayaz et al. 2015).

Purification of the lipase

The *S. violascens* OC125-8 strain was incubated for 96 h in ISP_2 broth and the extracellular enzyme was isolated by centrifugation. The lipase was partially purified using ammonium sulphate precipitation and approximately 3.28-fold purification with 56% recovery was obtained; the specific activity of *S. violascens* OC125-8 was 146.4 U/mg. In another study, *Streptomyces* sp. AU-1 lipase was partially purified to 1.62-fold purification with 0.26% recovery using ammonium sulphate precipitation and gel-filtration chromatography (Sarac et al. 2017). Elsewhere, *Streptomyces* sp. OC 119-7 lipase was purified using similar methods to obtain 68.055 U/mg specific activity and 5.52-fold purification (Ayaz et al. 2015).

Characterisation of the lipase

Optimum substrate degradation by the lipase was achieved at 40 °C. The lipase performed highly, with degradations of 93.75% and 98.8% at 30 °C and 50 °C, respectively (Fig. 1). Previous studies have reported that optimal temperatures of lipases from *Streptomyces* are between 30 and 50 °C (Mander et al. 2012, 2014; Ugur et al. 2014; Ayaz et al. 2015; Sen et al. 2016; Yuan et al. 2016).

In temperature stability test, 87.5%, 86.8% and 74% of activity was retained after 1 h of incubation at 30 °C, 40 °C and 50 °C, respectively (Fig. 1). The high stability of the enzyme could make this lipase operable, particularly under ambient temperatures for use in wastewater treatment. Similarly, it has been reported that three lipases, isolated from different sources, optimally catalysed the hydrolysis of crude
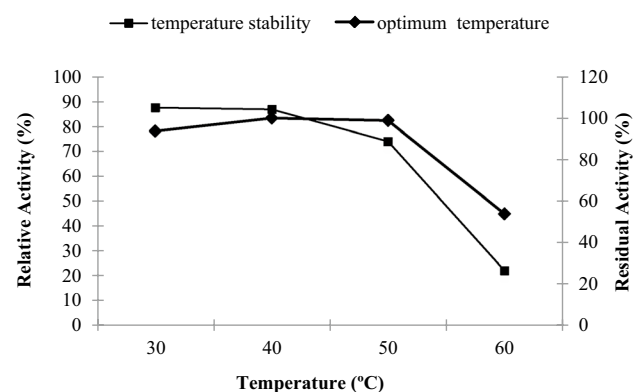

Fig. 1 Effect of temperature on lipase activity (line with diamond) and stability (line with square). Relative activity was measured under standard conditions and the maximum activity of the enzyme was taken as 100%. Residual activity was measured under standard conditions and initial lipase activity was taken as 100%

lipid waste at 40–50 °C and high temperatures caused a reduction in the rate of hydrolysis (Meng et al. 2017).

Optimum degradation of *p*-NPP by *S. violascens* OC125-8 was achieved at pH 8.0. The lipase also showed high degradation at pH 9.0 and 10.0 with efficiencies of 92.3% and 98%, respectively (Fig. 2). The results suggest that the bacterium possesses a high level of alkaline enzyme. Similar optimum pH profiles have been observed in *Streptomyces* sp. CS268, *Streptomyces* sp. CS273, *Streptomyces bambergiensis* OC 25-4 and *Streptomyces* sp. OC 119-7 (Mander et al. 2012, 2014; Ugur et al. 2014; Ayaz et al. 2015).

The operating pH range of most wastewater units is controlled in the range 6.5–8.5, depending on the wastewater and the target pollutant (Burgess and Pletschke 2008). Enzymes with stability over wide pH ranges are advantageous for wastewater treatment processes. The results of a pH stability test show that the *S. violascens* OC125-8 lipase was very stable across a broad pH range (pH 6.0–11.0) and that it maintained 71.74–100% activity after preincubation for 1 h (Fig. 2). Studies by *Streptomyces* also show that the pH stability of these bacterial lipases is generally neutral-alkaline. For example, the *Streptomyces* sp. OC 119-7 lipase exhibited stability at pH 7.0–9.0 (Ayaz et al. 2015); the *Streptomyces lienomycini* 350-2 lipase remained stable at pH 7.0–11.0 (Sen et al. 2016); the *S. bambergiensis* OC 25-4 lipase was stable at pH 6.0–7.0 (Ugur et al. 2014); and the *Streptomyces* sp. AU-1 lipase was stable at pH 10.0–11.0 (Sarac et al. 2017).

Kinetic analysis of the *S. violascens* OC125-8 lipase was performed with various concentrations of *p*-NPP as the substrate under optimal conditions. The kinetic parameters were calculated according to the Michaelis–Menten equation
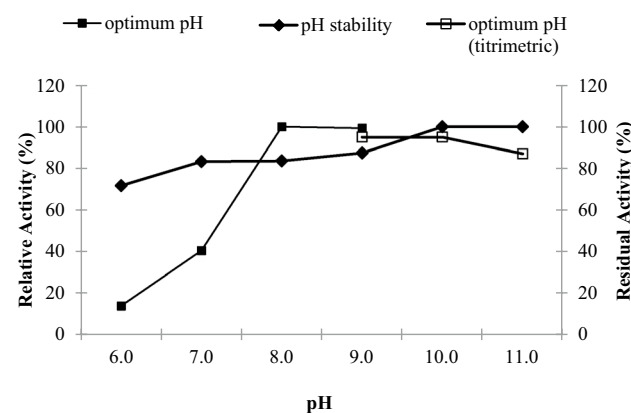

Fig. 2 Effect of pH on lipase activity and stability. Relative activity was measured spectrophotometrically (line with filled square) and titrimetrically (line with open square) and the maximum activity of the enzyme was taken as 100%. Residual activity (line with diamond) was measured under standard conditions and initial lipase activity was taken as 100%

from a Lineweaver–Burk plot. The V_{max} and K_m values were defined as 0.6 mmol/mg/min and 0.259 mM, respectively. In another study, the *Streptomyces* sp. CS268 lipase displayed highest hydrolytic activity in *p*-nitrophenyldecanoate with V_{max} and K_m values of 319.5 mmol/mg/min and 0.59 mM, respectively (Mander et al. 2012).The K_m value is the substrate affinity of an enzyme; a lower value emphasises high affinity between substrate and enzyme. A high V_{max} value indicates high catalytic performance by the enzyme.

Oily wastewaters are highly heterogeneous because they contain detergents, surfactants and metals, as well as various types of oil (Karhu 2015). For this reason, the effects of different metal ions, boron compounds, inhibitors, surfactants and commercial detergents on the stability of the *S. violascens* OC125-8 lipase were studied. Results show that the enzyme was inhibited by Mg^{2+}, Mn^{2+}, Cu^{2+} and H_3BO_3, while it was activated by Fe^{2+} and borax (Table 1). A probable cause of this indication is that Fe^{2+} and borax bind to the active site of the lipase and induce conformational changes in the enzyme, thus improving stability. Conversely, other metal ions support a less stable conformation of this lipase.

In addition, it has been reported that metal ions may act as cofactors during catalytic processes as well as increase the solubility of oil and fat at the interface (Lesuisse et al. 1993). It has also been generally recorded that lipases are stimulated in the presence of Ca^{2+} and that this may cause structural changes rather than aid their catalytic roles (Dandavate et al. 2009). However, and in contrast, the stability of *S. violascens* OC125-8 in the current study was reduced in the presence of Ca^{2+} (61.5%). Similar results have been observed in *Streptomyces fradiae* var. k11 (Zhang et al. 2008), *Streptomyces* sp. CS133 (Mander et al. 2012) and *S. bambergiensis* OC 25-4 (Ugur et al. 2014).

Various boron compounds are used in personal care products, household detergents and industrial cleaners to bleach the stains, improve surfactant performance, provide alkali buffering, soften water and stabilise the enzymes (Roskill 2002; Ascherl 2014). In this study, the *S. violascens* OC125-8 lipase was quite stable in the presence of boron components except for boric acid (76.9%) (Table 1).

Changes in stability were observed in the presence of inhibitors to determine the nature of the *S. violascens* OC 125-8 lipase (Table 1).The results show that the lipase displayed high activity in the presence of a chelating agent (EDTA) and a denaturing agent (SDS). The fact that *S. violascens* OC 125-8 is not affected by EDTA indicates that it is not a metalloenzyme. Furthermore, PMSF inhibited the lipase which proves that it belongs to the serine hydrolase class (Van Oort et al. 1989). EDTA and PMSF have been observed to have similar effects on other *Streptomyces* lipases (Mander et al. 2012; Ugur et al. 2014).

Among the oxidising agents studied, sodium perborate stimulated enzyme activity (114%), sodium hypochlorite

Table 1 Effects of metal ions, boron compounds, enzyme inhibitors, oxidising agents, surfactants and commercial detergents on *S. violascens* OC125-8 lipase activity

Residual activity (%)		Residual activity (%)	
Control	100 ± 0.01[a]	Control	100 ± 0.21[a]
Metal ions (5 mM)		Oxidising agents (0.1%)	
Ca^{2+}	61.5 ± 0.01	Hydrogen peroxide	25 ± 0.01
Cu^{2+}	38.4 ± 0.07	Sodium hypochloride	70.6 ± 0.03
Mg^{2+}	30.7 ± 0.2	Sodium perborate	114 ± 0.66
Mn^{2+}	40 ± 0.012		
Fe^{2+}	230 ± 0.87	Surfactants (1%)	
		Tween-40	111.7 ± 0.8
Boron compounds (5 mM)		Tween-60	110.8 ± 0.5
H_3BO_3	76.9 ± 0.01	Tween-80	108 ± 0.5
BKO_2	100 ± 0.09	Triton X-100	108.9 ± 0.031
$NaBO_2$	102 ± 0.32		
$Na_2B_4O_7$	105 ± 0.24	Commercial detergents (1%)	
		Omo	113.4 ± 0.05
Enzyme inhibitors (0.1%)		Ariel	128.3 ± 0.01
SDS	131 ± 0.1	Tursil	88 ± 0.07
PMSF	0 ± 0.0	Pril	97 ± 0.1
EDTA	104 ± 0.35	Fairy	98.5 ± 0.01

S. violascens OC125-8 lipase was incubated with various metal ions, boron compounds, enzyme inhibitors, oxidising agents, surfactants or commercial detergents at room temperature for 1 h before the activity was measured with *p*-NPP. Residual activity of enzyme without any additives was defined as 100%

[a]Values are expressed as mean $\pm$ standard deviation

slightly inhibited (70.6%) and H_2O_2 strongly affected (25%) the lipase activity (Table 1).

Surfactants decrease the interfacial area tension between water and oil and increase access to the substrate by the enzyme (Ogino et al. 2000). However, different surfactants may have different influences on the efficacy of the lipase. In this study, the *S. violascens* OC125-8 lipase was resistant to all surfactants used (Table 1). In contrast, it has been reported that the *Streptomyces* sp. CS268 lipase was inhibited slightly by Tween-20 and Tween-80 and was activated by Triton-X 100 (Mander et al. 2012). In another study, Tween-20, Tween-40 and Tween-80 stimulated activity of the *Streptomyces* sp. OC 119-7 lipase (Ayaz et al. 2015). The cause of the stimulatory impact of surfactants may be their action on the insoluble substrate (Colla et al. 2010).

The wastewater contains oil and fat as well as commercial detergents. In the presence of Ariel, Omo, Fairy and Pril, the *S. violascens* OC125-8 lipase retained 128.3%, 113.4%, 98.5% and 97% of its activity, respectively (Table 1). The *Streptomyces fungicidicus* RPBS-A4 lipase was also stable in Ariel (86.64%), Surf Excel (83.28%), Tide (80%), Nirma (76.64%) and Wheel (76.64%) after 1 h of incubation (Rajanikanth and Damodharam 2017). Similar results are presented elsewhere by Tang et al. (2017) in which the recombinant *Streptomyces* sp. lipase remained stable in Amway laundry detergent, Walch liquid soap and Super natural soap powder.

The natural oil hydrolysis of *S. violascens* OC125-8 lipase was determined titrimetrically using different oils as substrates; oil reduction by the lipase is presented in Fig. 3. Results show that the lipase has high affinity for natural oils; the highest degradations were observed in olive oil and sunflower oil at 100% and 90%, respectively. The burned sunflower oil and corn oil were less hydrolysed. A number of existing studies have reported on other lipases that are also able to degrade oils; *Serratia grimesii* RB06-22 lipase showed highest hydrolytic activity towards soybean oil and sunflower oil (Ugur and Boran 2014), and *Burkholderia* sp.
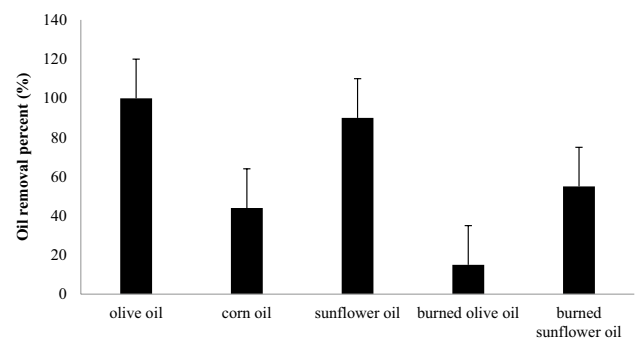

Fig. 3 Oil removal performance of *S. violascens* OC125-8 lipase. Free fatty acids were titrated with 0.05 M NaOH using phenolphthalein as indicator. Superscript a: values represent the means $\pm$ standard deviation

EQ3 lipase obtained from wastewater showed high activity towards corn oil and olive oil, while sunflower oil was less hydrolysed (Ungcharoenwiwat and Kittikun 2015).

Leal et al. (2006) used lipases in the biological treatment of grease and oil in dairy wastewater. Similarly, Meng et al. (2017) used three lipases (Lipase-I, Lipase-II and Lipase III) for the treatment of food wastes (animal fat, vegetable oil, and floatable grease). That study reported that lipid content in the waste was sufficiently hydrolysed after 24 h (in the range of 12.9–86.0%). Nevertheless, no study has investigated the potential of *Streptomyces* lipases for the targeted treatment of oily wastewater, although *Streptomyces* sp. AU-1 has been suggested as a potential biocatalyst in biodiesel production, showing high affinity to olive oil, sunflower oil and waste oil (Sarac et al. 2017).

Conclusions

In recent years, safe, efficient and cost-effective methods have been sought to remove oil waste and pollutants from the environment. Consequently, various studies describe the use of laboratory-developed microorganisms or their enzymes for the treatment of wastewater with high oil content; it is important to select stable and active enzymes at appropriate pH and temperature levels to significantly hydrolyse the wastewater. In this study, the *S. violascens* OC125-8 lipase displayed optimum substrate degradation activity at pH 8.0 and 40 °C. The enzyme remained stable in a wide range of pH values and ambient temperatures. In addition, lipases should exhibit strong stability in the presence of chemical substances and have affinity to ester chains of different lengths. The stability of *S. violascens* OC125-8 in the presence of various chemical substances including metal ions, surfactants, oxidising agents and commercial detergents and its affinity for different lengths of ester chain, indicate that it can be used to hydrolyse oily substances in wastewater treatment processes.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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