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Dear Editor in chief

Subject: Submission of a manuscript for publication in “**World Journal of Microbiology & Biotechnology**”

Sir,

I am attaching my manuscript entitled “Purification and Characterization of lipase enzyme from endophytic *Bacillus pumilus* WSS5 for application in detergent industry.” for publication in your esteemed Journal namely “**World Journal of Microbiology & Biotechnology**”.

The manuscript is about the purification and characterization of the lipase from an endophytic bacteria isolated from the stem of *Withania somnifera* L. Dunal. Endophytes have long been acknowledged as enzyme manufacturers for their natural requirements, more specifically as manufacturers of a sequence of enzymes needed to penetrate and colonize their plant hosts, including hydrolytic and oxidative enzymes, however, the possible use of endophytes as sources of industrial enzymes has not received much attention. The current findings suggest the potential of *B. pumilus* WSS5 lipase owing to promising properties such as the alkali-thermostability, organic solvent-tolerance, and broad substrate specificity that makes it a strong candidate for application in detergent formulations, biotransformation, industries, and medicine and moreover this is the first report on the purification and characterization of lipase from bacterial endophyte isolated from the stem of *Withania somnifera* L. Dunal. The authors have declared no competing interests. The data are original, no duplication and plagiarism, also have not published in elsewhere.

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Purification and Characterization of lipase enzyme from endophytic *Bacillus pumilus* WSS5 for application in detergent industry.

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Abstract

Lipolytic enzymes from endophytic microbes have been the focus of intense and growing research in view of the applicability of such enzymes in various industrial applications and endophytic microorganisms may emerge as a potential source of lipases with distinct characteristics. The current work involved purification and characterization of the lipase from an endophytic-bacteria isolated from the stem of *Withania somnifera* L. Dunal. Based on the analysis of morphology and 16S rDNA sequence, the endophytic bacteria was identified as *Bacillus pumilus* and designated as *B. pumilus* WSS5. Purified lipase from the *B. pumilus* WSS5 was found to be of 28 KDa as revealed by SDS-PAGE with optimum activity at 37°C and pH 8. The enzyme was found to be stable over a broad pH range of 6.0–9.0 and temperature stability was in the range of 10°C to 60°C. The enzyme activity was enhanced in the presence of organic solvents like Ethyl acetate, Methanol, 2-propanol, acetone, and ethanol. The stability of the enzyme in presence of various metal ions, surfactants, inhibitors, and kinetic parameters value suggested that this enzyme could be exploited in leather and detergent industries.

In addition, the stain removal potential of the crude and purified lipase was determined on cotton fabric pieces stained with vegetable oil. Lipase added to water along with detergent significantly enhanced the stain removal efficiency of a commercially available detergent. The current findings suggest the potential of *B. pumilus* WSS5 lipase owing to promising properties such as the alkali-thermostability, organic solvent-tolerance, and broad substrate specificity that makes it a strong candidate for application in detergent formulations, biotransformation, industries, and medicine. To our knowledge, this is the first report on the purification and characterization of lipase from bacterial endophyte isolated from the stem of *Withania somnifera* L. Dunal.

Keywords: Bacterial endophytes, *Withania somnifera* L. Dunal., *Bacillus pumilus* WSS5, lipase.

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Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and material

Data included in this article.

Code availability

Not applicable.

Authors' contributions

SR designed and supervised the study, SSK conducted the experimental work and compiled the manuscript. SSK, VV and SR analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The data are original, no duplication and plagiarism, also have not published in elsewhere.

Introduction

Lipases (EC 3.1.1.3) are triacylglycerol acylhydrolases that catalyze the breakdown of triacylglycerol to diacylglycerols, monoacylglycerol, fatty acids, and glycerol on the interface between aqueous and lipid phase, are widely present in plants, animals, and microorganisms (Nema et al. 2019). Lipases obtained from microorganism are most interesting due to the fact that these are often more useful than plant or animal-derived lipases because of the wide range of catalytic activities, easy genetic manipulations, regular supply due to lack of seasonal fluctuations, high yields, and rapid growth of microorganisms on affordable media (Arpigny and JAEGER 1999; Fang et al. 2006; Hasan et al. 2006).

Lipases have emerged as one of the leading biocatalysts in recent years, accounting for almost 10% of the enzyme market, and have shown the ability to add to the multibillion-dollar market share of the bio-industry. These are used as flavor-modifying enzymes in food additives, cleaning agents in detergents, pitch control in the paper, digestive enzymes in pharmaceutical products, etc (Carvalho et al. 2015; Guerrand 2017; Kumari et al. 2019; Raveendran et al. 2018; Salihu et al. 2016; Singh et al. 2016). Among the microbial sources of unique enzymes, the intent for screening bacterial endophytes as a prospective source of such enzymes refers to the hypothesized mutualistic connection between the endophyte and its host plant.

Endophytic bacteria can be explored as a new source for the production of various enzymes (Strobel 2003; Suto et al. 2002). Furthermore, endophytes have long been acknowledged as enzyme manufacturers for their natural requirements, more specifically as manufacturers of a sequence of enzymes needed to penetrate and colonize their plant hosts, including hydrolytic and oxidative enzymes, however, the possible use of endophytes as sources of industrial enzymes has not received much attention (Corrêa et al. 2014). There are many reports on the isolation of endophytes and different bioactive molecules from *W. somnifera* (L.) Dunal but endophytic bacteria associated with this plant have not been explored for the production of enzymes to a satisfactory level (Pandey et al. 2018; Qadri et al. 2013). Therefore, *W. somnifera* (L.) Dunal. can be considered as a source of endophytic bacteria serving an end number of enzymes that find their use in industrial processes.

In view of the continuous demand for lipases to be used for various industrial applications, the present study was undertaken with the main aim of purification and characterization of the lipase obtained from the endophytic bacterial isolate *Bacillus pumilus* WSS5. The application of lipase as a laundry additive was checked for determining the oil-destaining potential of the enzyme.

Materials and Methods

Collection of the Plant material

Selected plant parts like stem and leaves of an ethnomedicinal plant, *W. somnifera* L. Dunal, were collected from Shri Mata Vaishno Devi University botanical garden, Katra (32.9915° N, 74.9318° E) of Jammu & Kashmir, India. The collected samples were stored in sample bags and taken to the laboratory for further processing.

Isolation of endophytic bacteria

The stem and leaves of the explant were surface sterilized using the procedure described by Tiwari et al. 2010 with few modifications. To check whether the surface sterilization process was successfully done, the sterile distilled water used in the final rinse of explants was also plated on the same growth medium. The explants were placed on different media such as Luria Bertani Agar, water agar, and Nutrient agar. The plates were incubated at 37°C for 48 hours. The streak plate technique was used to purify them up to a single strain. All studies were conducted in triplicates. The isolated bacterial endophytes were preserved by lyophilizing the culture.

Screening of isolated bacterial cultures for lipase activity

Plate assay

All endophytic bacterial isolates were screened for the production of esterase/lipase activity on tributyrin agar (TBA) (Dutra et al. 2007) and rhodamine olive oil (ROA) agar plates (Kouker and Jaeger 1987). Lipase producing strains were identified after incubation for 48 h at 37°C.

Extraction of lipase

Based on the activity observed on plates one of the lipase producing strain WSS5 was taken for further studies. The strain was grown in the Luria Bertani broth and incubated for 24, 36, and 48 hours at 37°C. The Overgrown culture was centrifuged at 10,000 x g for 30 min in a refrigerated centrifuge. The resulting supernatant contained extracellular lipase was separated from the cell pellet

and the pellet was further processed for the extraction of an intracellular fraction. The pellet was resuspended in lysis buffer (0.05 M phosphate buffer, pH 7) and subjected to five rounds of cellular disruption (1 min) with the sonicator at 15 kHz for maximum enzyme recovery. The cell suspension was centrifuged (14000 x g for 30 min) and the cell-free extract was collected (intracellular lipase) and the enzyme activity of both the fractions (extracellular and intracellular) was checked.

Identification of the Bacterial Endophyte

Standard tests according to Bergey's Manual of Determinative Bacteriology 1994 were performed for the identification of the selected strain (WSS5). The genomic DNA of endophytic bacteria (WSS5) was extracted using the modified CTAB method (Doyle and Doyle, 1987) and the PCR amplification of 16S rDNA of the selected strain was done using bacterial universal primers, 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT-3') (Lane 1991). Amplification was done in an automated thermocycler (Eppendorf) with amplification steps as Preheating at 94°C for 3 min followed by 29 cycles with a denaturation step at 94°C for 30 sec, annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min, followed by final extension at 72°C for 10 min. The PCR product was purified using a Qiagen PCR Purification kit following manufacturer guidelines and was sequenced. To identify the closest neighbor of the selected isolate, their nucleotide sequences were subjected to Blast search in the NCBI GenBank database. A phylogenetic analysis was carried out by obtaining related sequences from the NCBI and an evolutionary tree was created using the neighbor-joining analysis method and MEGA software, version 6.0, with 1000 bootstrap values (Tamura et al. 2013).

Lipase assay

The lipase activity was determined using the modified titrimetric and spectrophotometric (using p-nitrophenyl caprylate as a substrate) method (Beisson et al. 2000; Blake et al. 1996).

Protein estimation and Purification

Protein estimation was performed as per the procedure provided by the Quantipro BCA assay kit (Sigma). A two-step purification technique was used to purify the enzyme. The concentrated solution was precipitated with ammonium sulfate and the precipitate was centrifuged at 12,000g and 4°C for 30 min. The pellet was re-suspended for the solubilization of the protein in a 100 mM

phosphate buffer (pH 7.0). This solution was loaded on a Hiload 16/10 Phenyl-Sepharose High-Performance column (M/s. GE Healthcare) which was preequilibrated with 25 mM Tris-HCl buffer pH 6.8 containing 1.0 M ammonium sulfate. The lipase was eluted using the 100mM sodium phosphate buffer (pH 7.0). The resulting fractions were checked for lipase activity by the Spectrophotometric method using p-NPC (p-nitrophenyl caprylate) as the substrate and the protein content was estimated by the BCA assay kit. The crude and partially purified lipase preparations were electrophoresed on SDS-PAGE gel (10% polyacrylamide) according to the method of Laemmli (Laemmli 1970) along with standard protein molecular weight marker of broad range from 3.5 KDa to 205KDa (GeNEI) and the molecular weight of the enzyme was calculated. The fraction rich in lipase activity was combined with 5% lactose (w/v) and was lyophilized to form a powder that was stored at 4°C for further analysis.

Detection of lipase activity by Zymogram

Lipase activity was detected in native PAGE by activity staining following the Febriani method with slight modifications (Febriani et al. 2013). Under non-denaturing conditions, the sample was run on 10 % polyacrylamide gels in a cold room. After electrophoresis, the gel was soaked in 50mM Tris buffer (pH-6.8) with 1% triton X -100 for 4 hrs at 4°C. After 4 hrs the gel was washed with distilled water and the gel was again soaked in 50 mM Tris buffer (pH-6.8) containing alpha naphthyl acetate (30mg/ml) and fast blue RR salt (20mg/ml). The gel was incubated for 30 min in dark at 37°C and the activity was shown as red bands.

Characterization of the purified lipase enzyme

Effect of temperature and pH on activity and stability of the enzyme

The optimum pH of the purified lipase enzyme was investigated by performing enzymatic hydrolysis reactions at various pH levels (4.0-11.0). The pH was maintained using different buffers: 0.1 M sodium citrate (pH 4.0–6.0), 0.1 M phosphate buffer (pH 6.0–8), 0.1 M Tris-HCl (pH 7.0–9.0) and 0.1 M glycine-NaOH (pH 9.0–11.0), respectively. The stability of the enzyme at different pH was studied by incubating the enzyme (without substrate) at different pH values ranging from 4.0 to 11.0. The optimum temperature for the determination of lipase enzyme activity was tested at a temperature range from 4-70°C. Similarly, for finding out the temperature stability of the lipase enzyme the

purified protein was incubated for 30 minutes at different temperatures ranging from 4-70°C, and the residual enzyme activity was measured spectrophotometrically.

Effect of inhibitors and Metal ions

The effect of various inhibitors like EDTA, DMSO, β -mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), and DTT on the activity of the purified enzyme, was estimated by calculating the enzyme activity after incubation with 2mM concentration of inhibitors at 30°C (pH 7.0) for 30 min under standard assay conditions. The influence of metal ions (Ca^{2+} , Na^+ and Mg^{2+}) was also observed by incubating the purified enzyme with 2mM concentration of salt solutions for 30 minutes at 30°C. After 30 minutes, the sample was collected to estimate the lipase activity.

Effect of Surfactants

The effect of surfactants on lipase activity was analyzed using 0.1% SDS, Triton- X100, Tween 20, and Tween 80 at 30°C for 30 min and the activity was measured using standard assay conditions.

Effect of Organic solvents

The spectrophotometric assay was used to determine the effect of organic solvents on the enzymatic activity after pre-incubation of an enzyme (0.02ml) in presence of different percentages (10-100 %) of organic solvents viz chloroform, acetone, ethanol, methanol, ethyl acetate, and 2-propanol at 30°C for 30 minutes.

Substrate specificity

The substrate specificity of the purified enzyme was estimated spectrophotometrically using different pNP esters as substrates like pNP-butyrate, pNP-laurate, pNP-acetate, pNP-caproate, pNP-stearate, pNP-myristate, pNP-palmitate and, pNP-decanoate.

Determination of Kinetic Constants

The steady-state Michaelis–Menten kinetic constants of K_m and V_{max} were determined by Lineweaver–Burk plot using the reaction rate at varying substrate concentrations of pNP-Caprylate under standard assay conditions. The catalytic constant (K_{cat}) of the purified enzyme was calculated using V_{max} , molecular weight, and a known enzyme concentration.

Lipolytic activity for all the above assays (except substrate specificity) was determined using spectrophotometric method with p-nitrophenyl caprylate (p-NPC) as the substrate. All the experiments were carried out in triplicate and data is presented as mean values with calculated standard deviations.

Oil-destaining efficiency of the *B. pumilus* WSS5 lipase

The lipase from bacterial endophyte WSS5 was tested for its ability as a laundry additive to remove oil stains from the cotton cloth. Lipase as a detergent additive was assessed for its washing performance on white cotton cloth pieces (5x5cm) that were stained with vegetable oil. The stained pieces of cloth were taken in separate Petri plates and the series of washing performances were done with and without enzyme to analyze the efficacy of lipase in removing stains from the clothes.

Results

Isolation and screening of endophytic bacterial isolates

In the present study, eighteen bacterial endophytes were isolated from the leaves and stem of *W. somnifera* L. Dunal. All isolated bacterial endophytes were subjected to rapid screening for the lipase activity using tributyrin and olive oil agar plates. In the screening test, strain WSS5 showed the highest lipolytic activity therefore, it was selected for further studies (Fig. 1).

Identification of the bacterial endophyte

Based on the biochemical tests and microscopic appearance, the isolated strain WSS5 was identified as *Bacillus* genus and further confirmation was done by the 16S rDNA sequence analysis (Table 1). A band of 1.5 Kb was obtained from PCR amplified product of total genomic DNA of WSS5 (Fig. 2 (A)). To infer its phylogenetic position, the sequenced 16S rDNA region of the isolate was compared to the sequences in the NCBI database through BLAST search analysis (<http://www.ncbi.nlm.nih.gov/>). The results reveal that the WSS5 bacterial endophyte exhibits a higher level of 16S sequence identity 97.69% to *Bacillus pumilus* (MK601669.1) and the evolutionary history was deduced using the Neighbor-Joining method (Figure 2 (B)). Therefore, the strain WSS5 was identified as *Bacillus pumilus* WSS5, and the sequence has been deposited in the Gen Bank under the accession number MK652854.1.

Purification of the enzyme

The Intracellular Lipase enzyme was extracted and purified from the bacterial endophyte *B.pumilus* using a two step purification protocol i.e. ammonium sulphate and precipitation hydrophobic interaction chromatography with Hiload 16/10 Phenyl-Sepharose High-Performance column. The lipase enzyme with a specific activity of 17.3 U/mg was purified from the cell-free extract with a purification fold of 8.2 (Table 2). Coomassie Brilliant Blue staining of the partially purified fraction obtained from phenyl sepharose chromatography showed that the purified enzyme migrated as a single band on SDS – PAGE indicating a molecular weight of approximately 28KDa (Fig.3). Zymogram analysis showed a single active lipase band (Fig. 4).

Characterization of the purified lipase enzyme

The colorimetric method was used to characterize the purified lipase since the titrimetric method is easily influenced by pH, temperature, and organic solvents.

Effect of temperature and pH on the activity and stability of the enzyme

The optimum temperature of the purified lipase enzyme was found to be 37°C. The enzyme was found to be remarkably stable from 10°C to 60°C however, a drop in enzyme activity was observed after 60°C (Fig. 5A). The optimum pH of the purified enzyme was found to be 8.0 and the enzyme was found to be stable in the pH range of 6.0 – 9.0. The results confirmed the alkaline nature of the enzyme (Fig. 5B).

Effect of inhibitors and Metal ions on the enzyme activity

The effect of inhibitors on lipase activity is shown in Fig. 6. Results show that the *B. pumilus* WSS5 lipase was completely inactivated by PMSF (Phenyl methyl sulphonyl fluoride) whereas β -mercaptoethanol, DTT (Dithiothreitol) and EDTA (Ethylenediaminetetraacetic acid) exhibits low inhibitory effect. However, ascorbic acid decreased the enzyme activity by 52% and the maximum activity was retained with ammonium persulphate. The metal ions had a negligible effect on the activity of the lipase enzyme, with 90-95% activity being retained in presence of the metal ions (Fig. 7). None of the metal ions increased the enzymatic activity of the lipase enzyme possibly confirming the non-metallo nature of the enzyme.

Effect of Surfactants

The effect of surfactants on the lipase activity of *B. pumilus* WSS5 lipase was checked and it was shown that the enzyme retained more than 90% of the activity in presence of most of the surfactants (Fig. 8).

Effect of Organic solvents

The effect of different organic solvents was tested on the activity of *B. pumilus* WSS5 lipase and it was observed that the lipase retained more than 90% of activity in presence of various organic solvents while the enzyme activity decreased considerably in presence of chloroform (Fig. 10).

Substrate specificity

Hydrolysis of fatty acid esters with different acyl chain lengths (C2–C18) was tested to determine the substrate specificity of the purified lipase. The *B. pumilus* WSS5 lipase effectively hydrolyzed substrates containing C2 to C18 fatty acids, indicating broad enzyme-substrate specificity. The short-chain fatty acids showed low activity resulting in 15% relative initial activity (Fig. 9). However, the initial hydrolysis of C4 to C10 fatty acid esters was approximately 90%.

Determination of Kinetic Constants

The Michaelis-Menten constant, maximum velocity (V_{max}), and catalytic rate constant (k_{cat}) of the purified enzyme from *B. pumilus* WSS5 was evaluated in presence of pNP- caprylate as substrate under optimal reaction conditions i.e. 30°C. The Lineweaver Burk plot displayed Michaelis-Menten behavior of the enzyme, and the K_m and V_{max} for pNP- caprylate were found to be 1.7 mM and 166.7 mmol/min/mg respectively, indicating a high catalytic efficiency and substrate affinity of the enzyme. Furthermore, k_{cat} value was found to be 8,335 sec⁻¹(Fig.11).

Oil-destaining efficiency of the *B. pumilus* WSS5 lipase

To determine the detergent potential of *B. pumilus* WSS5 lipase, simple cotton fabric pieces were stained with vegetable oil. The oil-stained cloth was subjected to various combinations of lipolytic treatments involving water, water with detergent, detergent water with the enzyme, and water with the enzyme. The oil-destaining activity was least for treatment with only plain water, suggesting its inefficiency in removing the stain. Treatment with water along with detergent alone did not promote the efficient removal of oil stains. However, complete removal of stain was observed when a combination of water with detergent along the purified enzyme was used (Fig 12).

Discussion

Endophytes in several unexplored environments around the globe have been intensively studied (Chauhan et al. 2019; Fouda et al. 2021; Hagh Doust et al. 2019; Kamat et al. 2020; Singh and Dubey 2018). A little work has been done in this area from Jammu & Kashmir region to exploit the potential of the hidden treasure of indigenous plant endophytes. In this study, we successfully isolated a novel lipolytic strain *B. pumilus* WSS5 from the *W. Somnifera* L. by tributyrin agar plate and olive oil agar plates screening and evaluated the properties of the corresponding lipase. The strain was identified as *Bacillus pumilus* by the 16S rDNA sequence analysis.

The protein was precipitated for purification purposes using ammonium sulphate salt since it is highly soluble in water, cheap, and had no deleterious effect on protein structure. The lipase enzyme was effectively precipitated at 60% ammonium sulphate concentration leading to a purification fold of 4.9. Our findings are in close agreement with the purification approaches adopted by Saun et al from *Bacillus aerius* (Saun et al. 2014) and Li et al. for the precipitation of the *Stenotrophomonas maltophilia* CGMCC 4254 novel cold-active and organic solvent-tolerant lipase (Li et al. 2013). The enzyme was partially purified using phenyl Sepharose chromatography and the results were confirmed by 10% SDS PAGE showing a single band indicating the purity of the enzyme and the molecular weight was found to be about 28 KDa. This result is similar to the molecular weight reported for lipase from other strains like *Staphylococcus epidermidis* strain L2, *Geobacillus sp.* 12AMOR1, and *Burkholderia cepacia* A.T.C.C. 25609 (Dalal et al. 2008; Edupuganti et al. 2017; Tang et al. 2019). Zymography was done using a non-denaturing gel with the purified lipase and a clear red band was observed which suggested a single enzymatically active lipase band.

The stability of enzymes with respect to temperature and pH is one of the critical parameters for their application in various industrial applications. Temperature plays a crucial role in determining the enzyme activity. The optimal temperature for the lipase activity was found to be 37 °C. Thereafter, the rise in temperature showed a gradual decline in the activity of the enzymes. The likely reason for this decrease could be the destruction of the tertiary structure of lipase, which would have altered the active site configuration and thus decreased the interaction of the enzyme substrates (Das et al.

2016). However the enzyme was found to be stable in a wide temperature range from 10- 60°C indicating a useful property for reactions performed at higher temperatures just like in detergent formulations, it could be a valuable asset. The present result is supported by Hu et al. (Hu et al. 2018); Jaiganesh and Jaganathan (Jaiganesh and Jaganathan 2018); Mohan et al. who recorded that the optimum temperature

for lipase activity from *Pseudomonas aeruginosa*, *Burkholderia sp.* and *Bacillus sp.* respectively was 35 °C (Mohan et al. 2008). Similarly, at optimal pH, enzymes are most active because their active sites have full interaction with the substrate. Every drastic change in the pH of the medium results in the denaturation of the enzyme leading to a loss of its activity. The optimum pH for enzyme activity in the present study was found to be 8.0 and the enzyme was stable in the pH range of 6-9. Based on its optimum pH value for lipolytic activity the enzyme can be considered as alkaline lipase. In many industrial applications, such as detergent formulations, sewage treatment, and leather processing alkaline lipases are essential (Salwoom et al. 2019). Similar results have been reported by different researchers (Femi-Ola et al. 2018; Kumar et al. 2020; Laachari et al. 2015; Mukesh Kumar et al. 2012).

Inhibitors are known as substances that decrease the activity of an enzyme-catalyzed reaction. They act by affecting the catalytic properties of the active site either directly or indirectly (Sharma et al. 2017). The reducing agent DTT and β -mercaptoethanol showed a low inhibitory effect. A similar observation was made by Zhao et al. and Das et al. (Das, Shivakumar, Bhattacharya, Shakya, Swathi 2016; Zhao et al. 2021). However contradictory reports have been reported in case of EDTA. In some cases it has stimulatory or no effect on lipase activity (Sahay and Chouhan 2018) whereas in others cases exhibit inhibitory effect (Sharma, Sharma, Pathania, Handa 2017). So in our case the enzyme retained 62% activity that means EDTA probably chelated the metal ions present with the enzyme, thereby denaturing the enzyme. In the presence of PMSF the activity of the purified lipase was completely lost suggesting the presence of serine residues in the active site of the enzyme as reported by various studies (Ekinici et al. 2016; Hu, Cai, Wang, Du, Lin, Cai 2018). The enzyme activity was not affected by the presence of different metal ions which concluded it as a non-metalloenzyme and this feature could be advantageous as the enzyme is not dependent on the presence of metal ions for

catalytic activity. Our results are consistent with a novel solvent stable lipase isolated from *Pseudomonas reinekei* (Priyanka et al. 2019).

Surfactants are known to minimize the interfacial tension between water and oil and improve the water-lipid interface area, which in turn increases the rate of lipase-catalyzed reactions (Shaoxin et al. 2007). The addition of 0.1% SDS, Tween-20, Tween-80, and Triton X-100 resulted in stable enzyme activity. Therefore the lipase activity remains active in the presence of both non-ionic and anionic surfactants. Our results are in close agreement with the studies of Das et al who has also reported the addition of detergents resulted in stable enzyme activity (Das, Shivakumar, Bhattacharya, Shakya, Swathi 2016).

The use of enzymes, especially lipases, in organic solvents is gaining considerable industrial importance since the process leads to the production of high-value products (Verma et al. 2008). In our study, we tested the activity of the lipase in six different solvents i.e. acetone, chloroform, ethanol, methanol, 2-propanol, and ethyl acetate. Maximum lipolytic residual activity was observed in Ethyl acetate followed by Methanol, 2-propanol, acetone, and ethanol. The enzyme showed high activity except for chloroform in all organic solvents. The activation of lipases in the presence of certain hydrophilic organic solvents can be explained by the interactions between certain amino acid residues and the organic solvent, shifting the lipase confirmation from the closed to the open shape, thereby increasing the lipase activity (Cao et al. 2012).

Substrate specificity of purified lipase was evaluated using pNP Esters of varying chain lengths. The maximum activity was found towards medium-length fatty acid esters (C4, C8, and C10) while as low activity was observed with the short-chain fatty acids. Thus the enzyme is more specific to soluble fatty acid esters of moderate chain lengths. Similarly, specificity for medium-chain fatty acids has also been observed for other purified Mucoromycota lipases, including the enzyme from *Rhizopus homothallicus* (Diaz et al. 2006), *R. miehei* (Takó et al. 2017), and *Rh. chinensis* (Sun and Xu 2008),

The K_m and V_{max} of the purified lipase enzyme was found to be 1.7 mM and 166.7 mmol/min/mg, using pNP- caprylate as substrate whereas K_{cat} was 8,335 sec⁻¹. The low K_m of *Bacillus pumilus* WSS5 lipase indicates a high affinity of this enzyme towards pNP-caprylate.

Therefore the low K_m value along with high K_{cat} values of the purified enzyme from *B. pumilus* WSS5 lipase could be beneficial both from the application and economical perspective.

In the present study, detailed analysis of the lipase enzyme from *B. pumilus* WSS5 confirmed it as an ideal candidate for use in laundry detergents as the enzyme was found to be very effective in the removal of oil stains from cotton cloth. Our results confirmed the use of this enzyme as an additive in detergents as the enzyme has broad stability towards temperature and pH and was found to be stable in presence of metal ions, solvents, and surfactants. The enzyme along with the detergents improved the efficiency of destaining keeping the color and quality of the fabric intact. Some studies have already reported such results but our results are more efficient than the previous ones by Hasan et al. and Sharma et al. (Hasan, Shah, Hameed 2006; Sharma, Sharma, Pathania, Handa 2017).

Thus, the results of this study suggest that *B. pumilus* WSS5 lipase has great potential as an additive in the detergent industry to eliminate oil stains at a wide temperature and pH range.

Conclusion

In the present work, an intracellular enzyme was obtained from a bacterial endophyte of the medicinal plant *Withania somnifera* L. Dunal of the J&K region. This intracellular lipase from *B. pumilus* WSS5 was purified and characterized and showed several properties suitable for different industrial applications. Since *B. pumilus* WSS5 lipase showed stability towards a wide range of temperature and pH, metal ions, surfactants, and organic solvents, this enzyme is a good candidate for detergent and other industrial applications.

Moreover, the lipase enzyme in the present study purified from endophytic *Bacillus pumilus* WSS5 is stable towards various organic solvent stable lipase and has wide substrate specificity suggesting its capacity as a biocatalyst for transesterification reactions. The Structural characteristics and cloning of the lipase gene in *E.coli* in future research will unveil further characteristics of the enzyme.

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Figure legends

Fig. 1: (A) Tributyrin Agar plate showing zone of clearance thus indicating lipolytic activity in *Bacillus pumilus* WSS5 (B) Olive oil Agar plate showing zone of clearance thus indicating lipolytic activity in *Bacillus pumilus* WSS5.

Fig. 2: (A) 1 % Agarose Gel showing PCR amplified product (M- 1 kb DNA ladder; 2- PCR amplified product of 16S rDNA from *Bacillus pumilus* WSS5).(B) A phylogenetic tree constructed by neighborhood joining method using MEGA6 with 16S rRNA gene sequences of different *Bacillus* spp. The filled circle in red represent the studied isolate and the outgroup, respectively.

Fig. 3: SDS-PAGE electrophoregram showing different fractions obtained during purification steps. Lane 2: Protein marker, Lane11: 60% ammonium sulfate precipitate, Lane6: Purified fraction from HIC, Lane 1, 3,4,7,8,9,10,12 &13: Empty .The molecular sizes of the marker protein are shown on the left.

Fig. 4: Zymogram analysis using a non-denaturing polyacrylamide gel with α - naphthyl acetate and fast blue RR salt for detection of lipolytic activity. The lipase activity resulted from zymogram analysis is shown in dark red color. Lane 1: HIC Purified fraction, Lane2: 60% pellet.

Fig. 5: Effect of Temperature and pH on Lipase enzyme activity of *Bacillus pumilus* WSS5 (A) Temperature Optima &Temperature Stability (B) pH Optima & pH Stability.

Results represented are the mean of 3 independent experiments and Standard Deviations are noted.

Fig. 6: Effect of different inhibitors at 2mM concentration on lipase activity of *Bacillus pumilus* WSS5. The control represents 100% of lipase activity in the absence of inhibitors and metal ions under the same condition. Results represented are the mean of 3 independent experiments and Standard Deviations are noted.

Fig. 7: Effect of different metal ions at 2mM concentration on lipase activity of *Bacillus pumilus* WSS5. The control represents100% of lipase activity in the absence of metal ions under the same condition. Results represented are the mean of 3 independent experiments and Standard Deviations are noted.

Fig. 8: Effect of Surfactants at 0.1% concentration on purified Lipase enzyme from *Bacillus pumilus* WSS5. The control represents 100% of lipase activity in the absence of surfactants

under the same condition. Results represented are the mean of 3 independent experiments and Standard Deviations are noted.

Fig. 9: Substrate specificity of purified Lipase enzyme from *Bacillus pumilus* WSS5. Results represented are the mean of 3 independent experiments and Standard Deviations are noted.

Fig. 10: Effect of various organic solvents on the stability of *Bacillus pumilus* WSS5 lipase.

Fig. 11: A Lineweaver Burk plot for the purified *Bacillus pumilus*WSS5 lipase using pNP-caprylate as a substrate over the substrate concentration range 1–20mM under standard assay conditions.

Fig. 12: Washing performance of *B. pumilus* WSS5 lipase on vegetable stained clothes. (A) Cloth stained with vegetable oil washed with distilled water, *B. pumilus* WSS5 and lipase detergent (B) Cloth washed with distilled water only (C) Cloth washed with detergent and distilled water (D) Cloth washed with *B. pumilus* WSS5 lipase and distilled water.

Fig. 1:

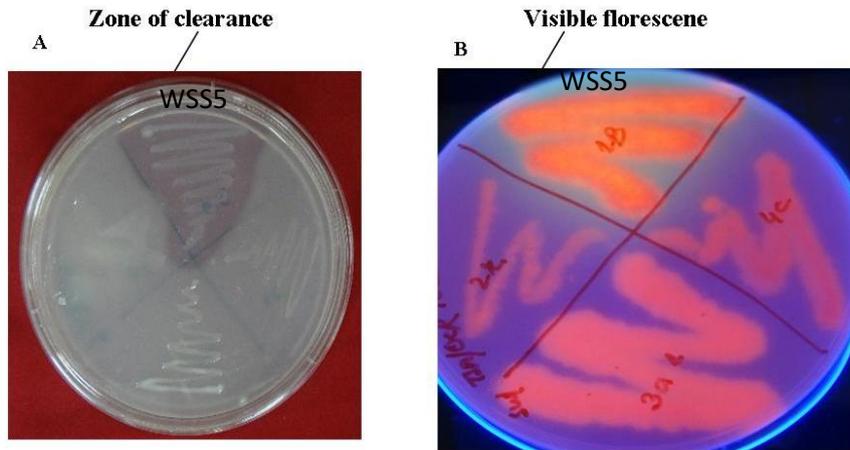
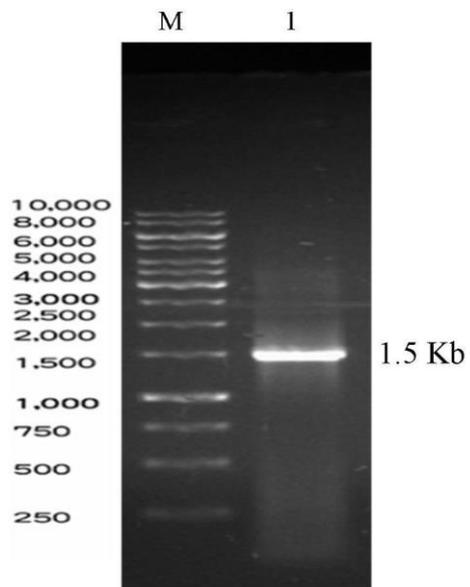


Fig. 2:

(A)



(B)

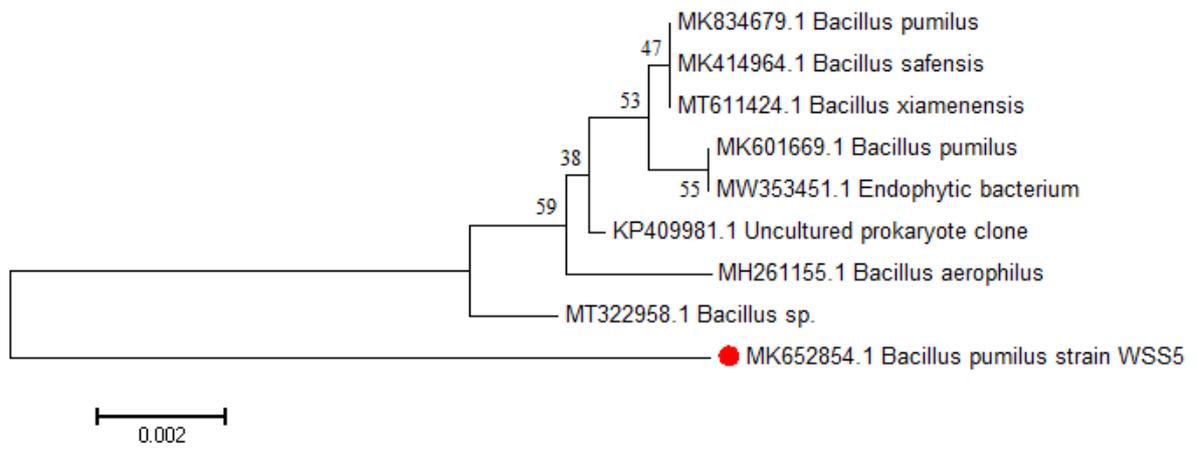


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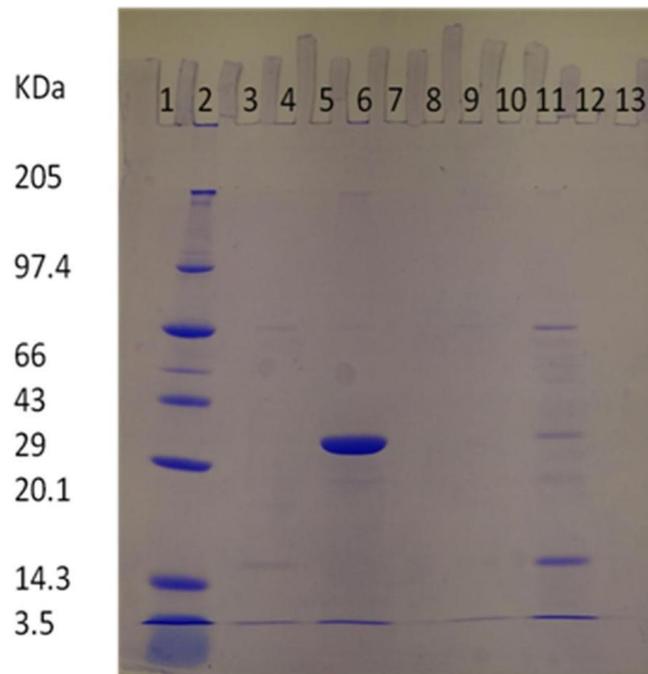


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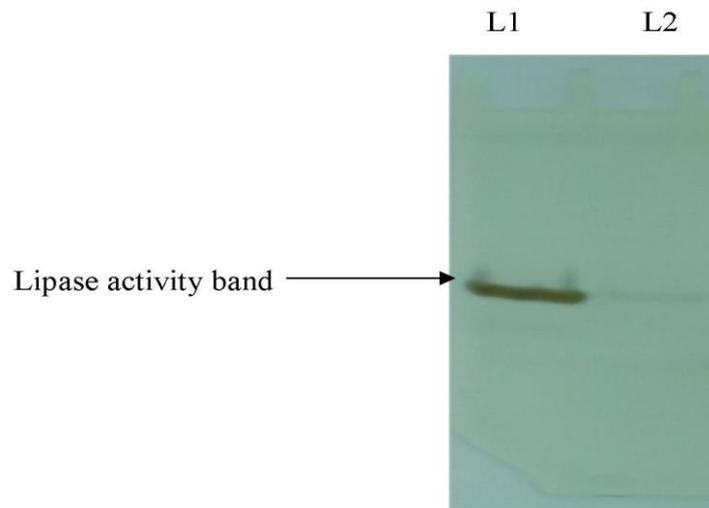
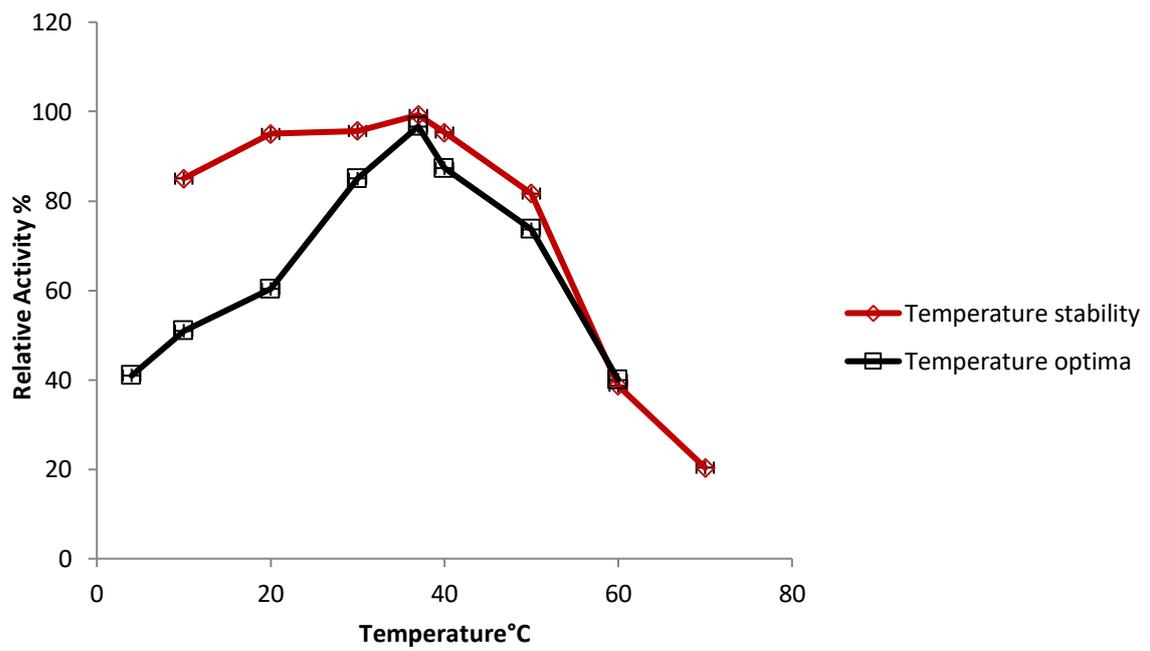
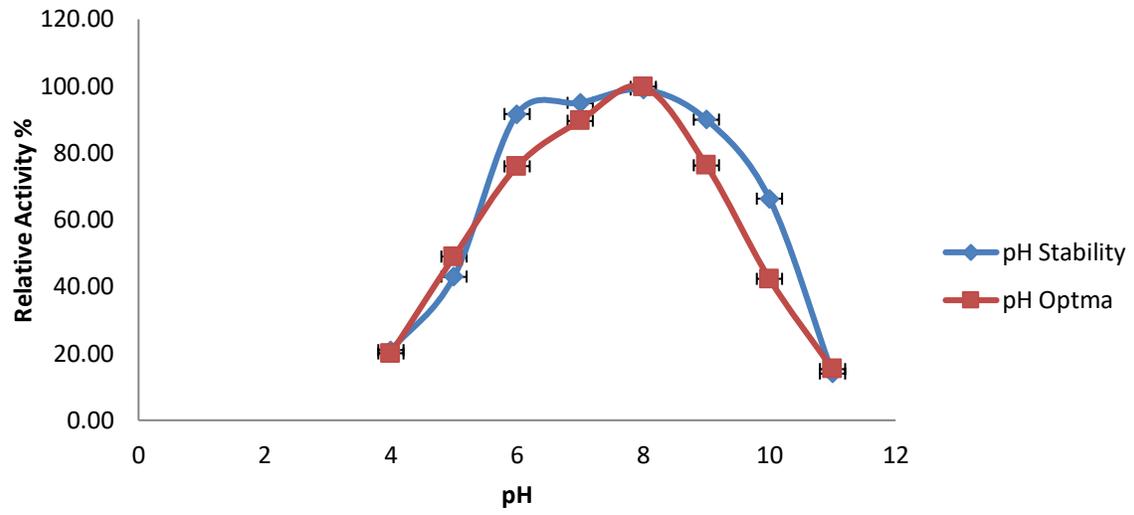


Fig. 5:



(A)



(B)

Fig. 6:

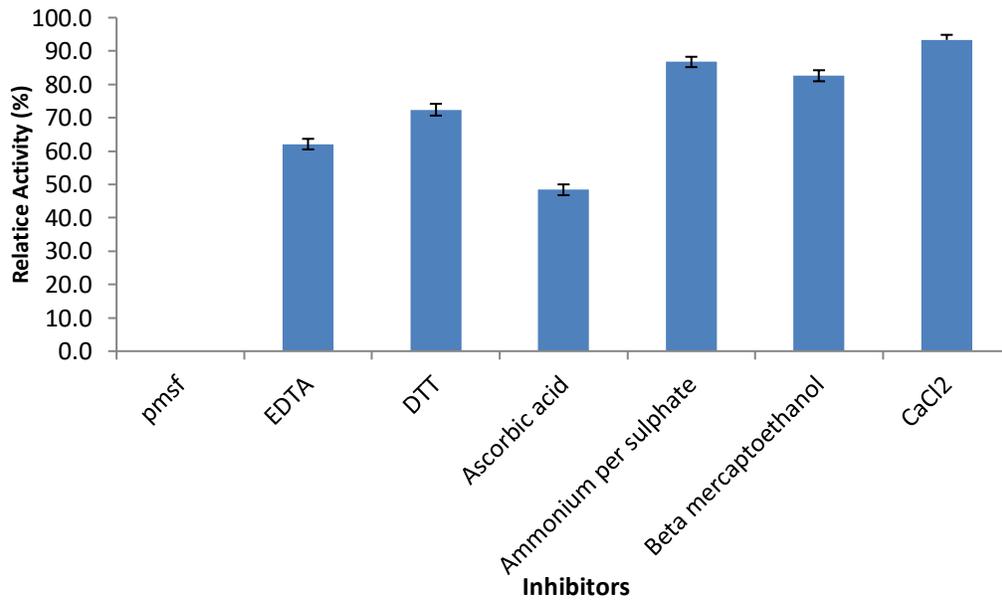


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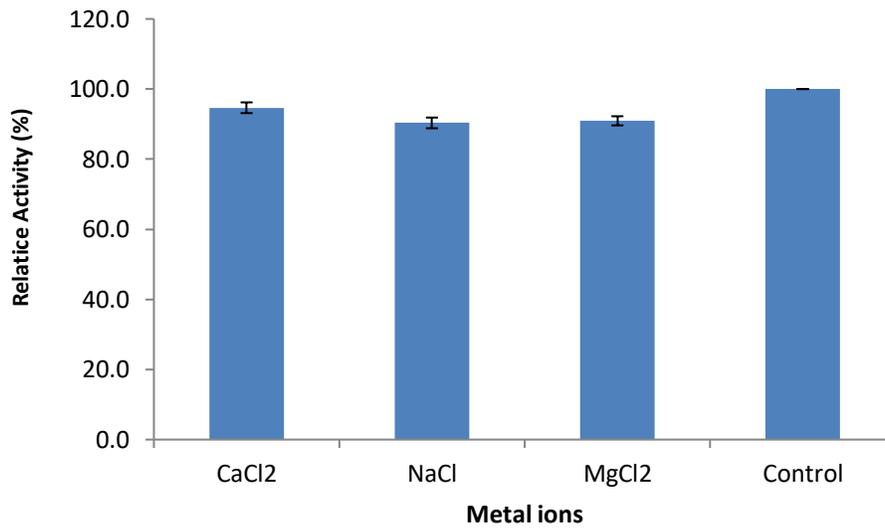


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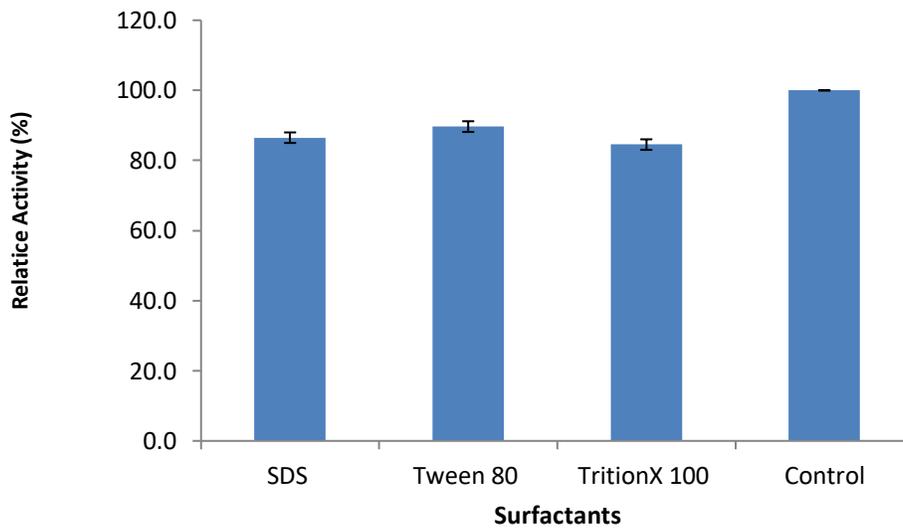


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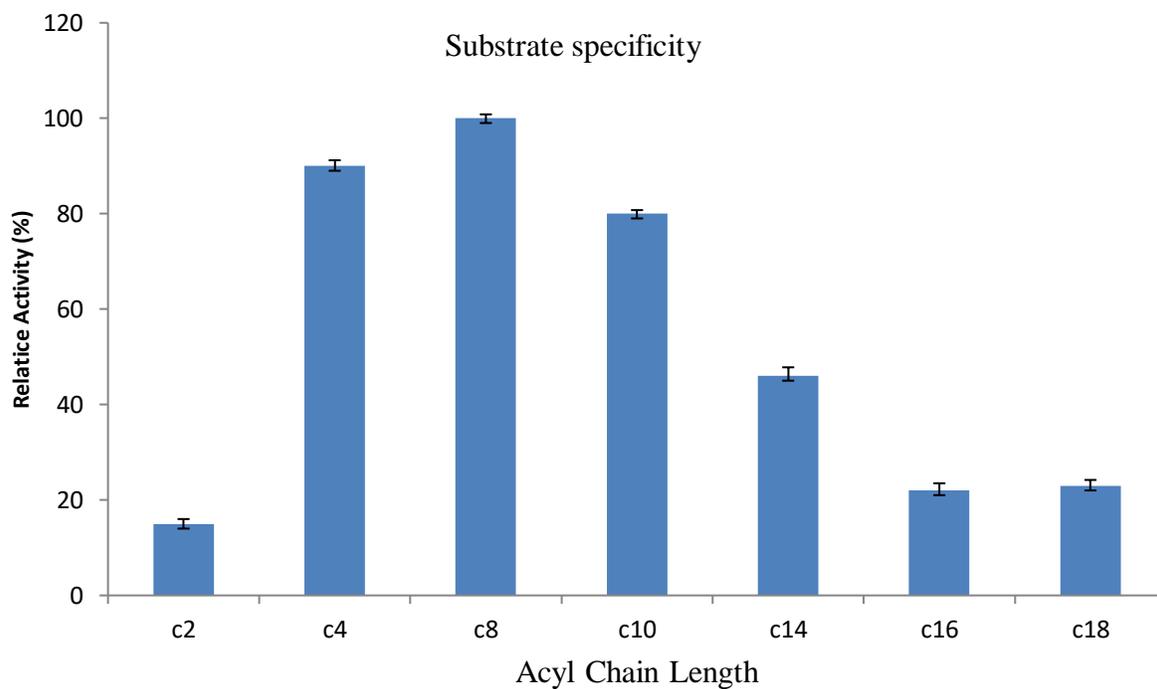


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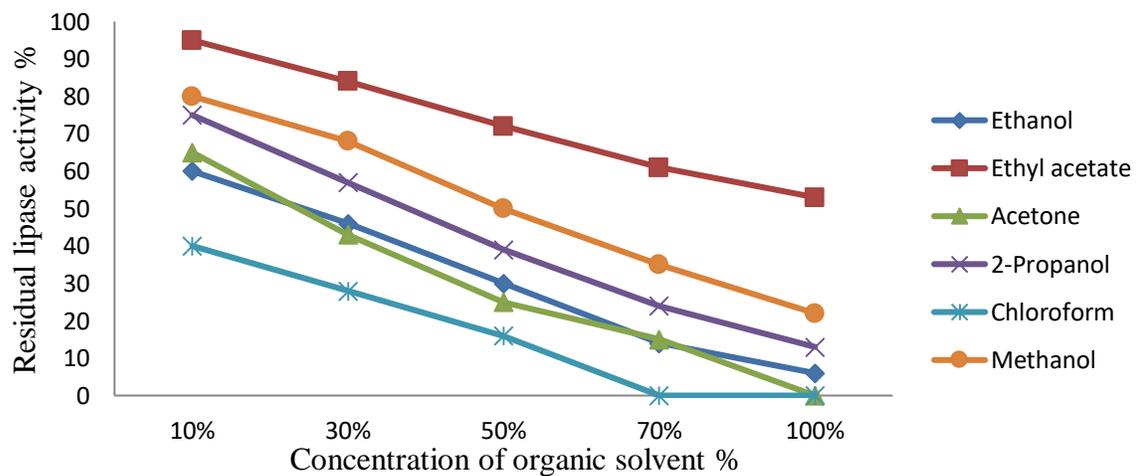


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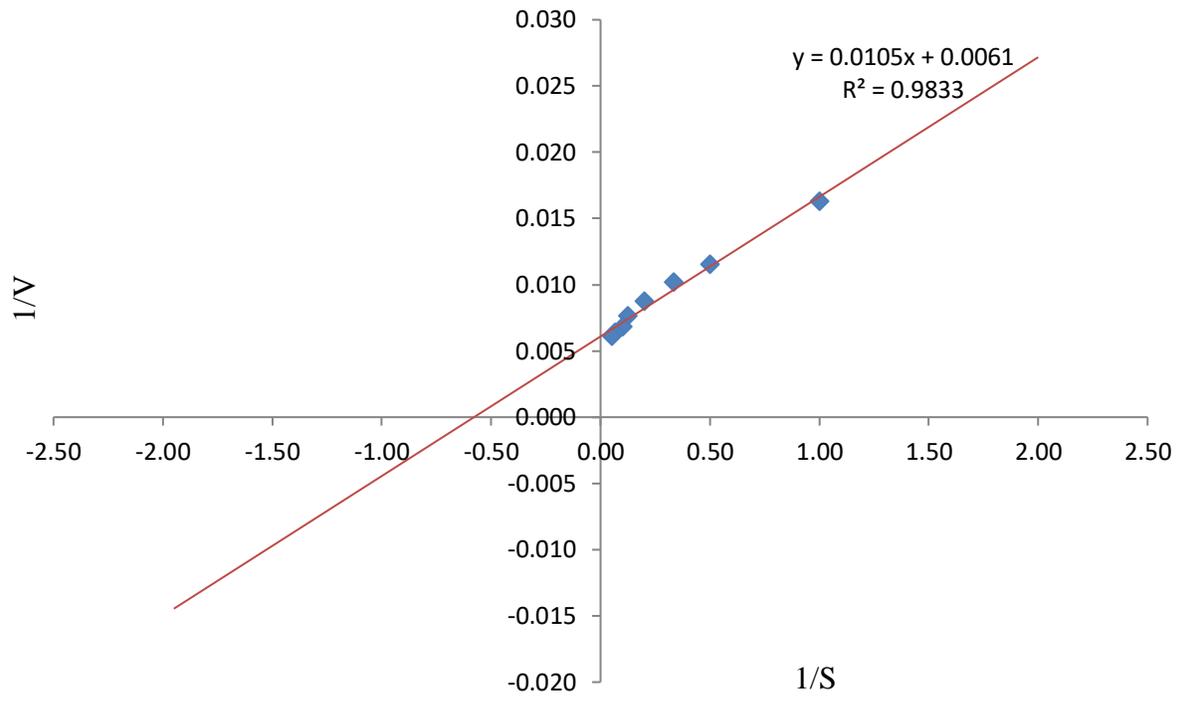


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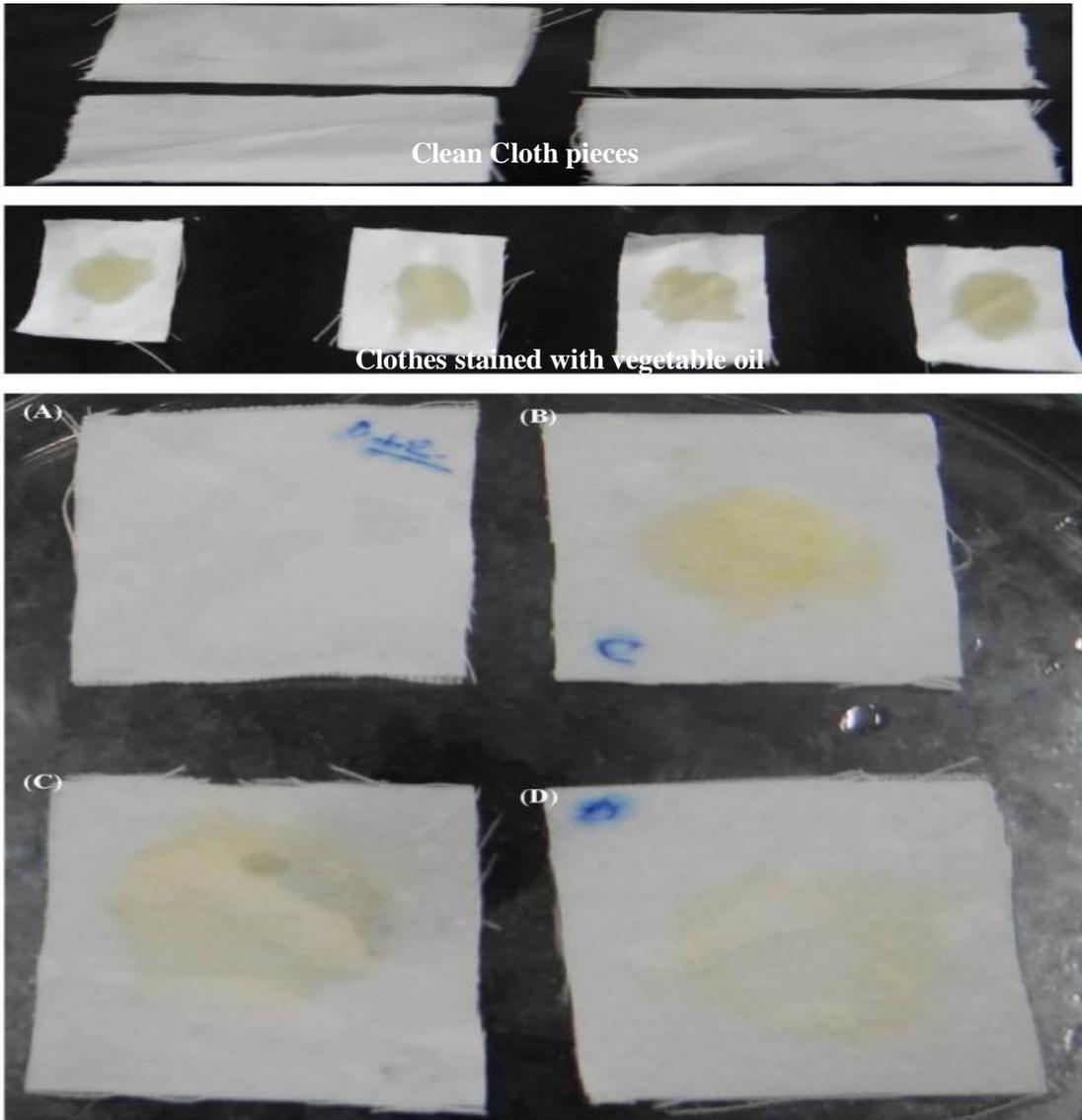


Table 1: Biochemical tests for the identification of endophytic bacteria WSS5 isolated from *Withania somnifera* L. Dunal.

S.NO	Biochemical Test	Results
1	Indole Production	–
2	Methyl Red Test	–
3	Voges Proskauer Test	+
4	Citrate Utilisation Test	-
5	H ₂ S production	–
6.	Simmon citrate agar	–
7	Catalase Test	+
8	Oxidase Test	+
9	Casein Hydrolysis	+
10.	MacConkey agar	–
12	Mannitol salt agar	+

“+” and “–” indicate positive and negative results, respectively;

Table 2: Table showing the summary of Purification of lipase from *Bacillus pumilus* WSS5

Sample	Total protein (mg/ml)	Total Activity (U)	Specific activity (U/mg)	Purification Fold	Yield (%)
Original	617	1317	2.1	1	100
60% Fraction precipitation	77.1	793.4	10.3	4.9	60.24
HiLoad 16/10 Phenyl-Sepharose column	27.3	473	17.3	8.2	35.9