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ORIGINAL ARTICLE

Molecular characterization of lipase from a psychrotrophic bacterium *Pseudomonas* sp. CRBC14

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Abstract

Lipases from *Pseudomonas* species are particularly useful due to their broader biocatalytic applications and temperature activity. In this study, we amplified the gene encoding wild-type cold-active lipase from the genome of psychrotrophic bacterium isolated from the Himalayan glacier. The isolated CRBC14 strain was identified as *Pseudomonas* sp. based on the 16S rRNA gene sequence. Lipase activity was determined by observing the hydrolysis zone on nutrient agar containing tributyrin (1%, v/v). The sequence analysis of cold-active lipase revealed a protein of 611 amino acids with a calculated molecular mass of 63.71 kDa. The three-dimensional structure of this lipase was generated through template-supported modeling. Distinct techniques stamped the model quality, following which the binding free energies of tributyrin and oleic acid in the complex state with this enzymatic protein were predicted through molecular mechanics generalized born surface area (MMGBSA). A relative comparison of binding free energy values of these substrates indicated tributyrin's comparatively higher binding propensity towards the lipase. Using molecular docking, we evaluated the binding activity of cold-active lipase against tributyrin and oleic acid. Our docking analysis revealed that the lipase had a higher affinity for tributyrin than oleic acid, as evidenced by our measurement of the hydrolysis zone on two media plates. This study will help to understand the bacterial diversity of unexplored Himalayan glaciers and the possible application of their cold-adapted enzymes.

Keywords Himalayas · *Pseudomonas* sp. · Cold-active lipase · MMGBSA · Flexible docking

Introduction

Lipases are the potential bioresources, mainly responsible for the hydrolysis of acylglycerides, while some are more suitable for synthesis (Kumar et al. 2020). Among all the lipases, cold-active lipases found naturally in psychrotrophic and psychrophilic bacterial species have gained a lot of attention in structural investigations and industrial applications due to their remarkable stability at low temperatures (Bhatia et al. 2020). These lipases have presented great activity in biofuel production (Ribeiro et al. 2011), as detergent additives (Al-Ghanayem and Joseph 2020), in environmental bioremediations and food industries (Chandra et al. 2020), leather processing and suppressing the formation of inclusion bodies in protein expression studies at low temperatures (Sathish Yadav et al. 2011; Joseph et al. 2008). Moreover, the global market for lipases is expected to reach $ 0.79 billion by 2025 (Fatima et al. 2020). Cold-active lipases are in growing demand (Mhetras et al. 2021) because they are active at low temperatures and low water concentrations due to improved flexibility compared to their mesophilic and thermophilic counterparts (Kumar et al. 2020).

Furthermore, microbial lipases have a lower cost of manufacture, a more selective activity, lower energy usage (Kavitha 2016), easy handling and transportation in powdered form in the enzyme market in comparison to lipases from plant and animal sources (Chandra et al. 2020).

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Pseudomonas lipases, in particular, have received special attention among bacterial lipases because of their thermoresistance, active at alkaline pHs and higher production rate (Chandra et al. 2020; Ramnath et al. 2016). According to their molecular properties and the need for correct folding and secretion of helper proteins, these lipases have been categorized into three groups viz., the groups I, II and III. Group III lipases do not require a lipase-specific foldase to obtain enzymatically active lipases and have a molecular mass between 50 and 68 kDa (Karakaş and Arslanoğlu 2020).

The common methods of identifying the new lipases isolated from natural sources such as plants, animals and microorganisms used established protocols or selected methods. These methods include fermentation, precipitation and purification of enzymes, which are usually time and resource consuming (Bharathi and Rajalakshmi 2019). Alternatively, the amplification of the target gene can be carried out using degenerate primers (Abd. Jalil et al. 2018). In addition, the *in silico* characterization of these enzymes also offers a higher success rate, increased discoverability and lower consumption of time and resources (Kamble et al. 2018). The discovery of new lipases through the combined molecular and *in silico* approach for industrial use has become a valuable tool due to the increasing availability of whole-genome sequences (Kamble et al. 2018). Thus, several lipase-encoding genes have either been amplified from the wild-type bacterial species or cloned into various other species (Baweja et al. 2016; Perfumo et al. 2020).

In light of this, there is greater interest in exploring cold habitats to isolate such enzymes from psychrotrophic bacteria for commercial purposes. Kashmir Himalaya, which lies to the north-western extremity of the Himalayan biodiversity hotspot, has been less explored for lipase-producing psychrotrophic bacteria (Yadav et al. 2016; Joseph et al. 2012). In this study, we used a combinatorial approach to characterize the cold-active lipase isolated from a psychrotrophic bacterium, CRBC14 of the Himalayan Thajwas glacier. Further, we explored the binding free energy of this lipase towards tributyrin and oleic acid through an extra-precision molecular docking approach in flexible mode and next-generation solvation model-based molecular mechanics generalized born surface area (MMGBSA) approach.

Materials and methods

Sample collection

The soil sample was collected at an altitude of 2944 m (34°16′30″N; 75°17′10″E) from the Himalayan Thajwas glacier. The soil sample was collected in 100 ml sterile plastic vials and transported to the laboratory in ice packs for analysis.

Isolation of the psychrotrophic bacteria

Isolation of bacterial colonies was carried on Luria–Bertani (LB) agar plates as per Srinivas et al. (2011). Soil sample (1 g) was dissolved in 100 ml of NaCl solution (0.9%, w/v) and kept in an orbital shaker incubator for 2 h at 150 rpm and 15 °C. Following that, 0.1 ml of the soil sample solution was inoculated on pre-prepared LB agar plates. The inoculated LB plates were then incubated at 4, 15, 20 and 30 °C for 2–15 days, colony counts were taken and distinct morphotypes were purified and kept on LB agar medium.

Screening for lipolytic activity

The isolated, pure colonies were screened for their lipolytic activity (clear zone) on nutrient agar (NA) plates containing tributyrin (1%, v/v). The strain CRBC14 with a maximum lipolytic activity was chosen for further analysis. The pure isolate was grown at five different temperatures (4, 10, 15, 20 and 30 °C) and pHs (6.0, 7.0, 8.0, 9.0 and 10.0) on two sets of NA plates. One set contained tributyrin (1%, v/v) and the other olive oil (1%, v/v) for the determination of lipolytic activity by analyzing the zone of hydrolysis (Joseph et al. 2012).

Determination of optimal growth temperature

The purified isolate was inoculated on pre-prepared LB plates and incubated at 7 different temperatures (0, 5, 10, 15, 20, 30 and 37 °C) to determine the optimal growth temperature. Growth was observed every 24 h for 2–30 days (Zhang et al. 2013).

Genomic DNA extraction and 16S rRNA gene amplification

The extraction of genomic DNA from psychrotrophic bacterial isolate CRBC14 was done by DNA purification kit (HiPura bacterial genomic DNA), as per the manufacturer's instructions. The extracted DNA was used as a template for 16S rRNA gene amplification through PCR using 27F and 1429R primers (Shivaji et al. 2004). The PCR product purification and sequencing were done at Agrigenome labs, Kerala, India. Identification of isolate (CRBC14) was done by BLASTn (Nucleotide BLAST) search at NCBI to check the similarity with our sequence. Further, the sequence alignment of CRBC14 with similar species (downloaded at NCBI) was done with ClustalW (https://www.genome.jp/tools-bin/clustalw). MEGA 7 was used to create the

phylogenetic tree (Kumar et al. 2016) by the Neighbor-joining method (Bootstrapping at 1000 replicates).

Amplification of lipase gene

Lipase gene was amplified by PCR using the primers based on known *Pseudomonas* lipase sequences: (LF) 5′-ATGGCT GTGTAGGACAAAAGAAC-3′ and (LR) 5′-TCAGGCGAT TACAATGCCATCAGC-3′ (Zhang et al. 2007). Protein sequences of cold-active lipase from CRBC14 strain and of other similar lipases were aligned together in Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and Fig. 2 was developed with ESPript 3.0.0 (Robert and Gouet 2014).

Model generation and assuring its validity

Using the amino acid sequence of cold-active lipase as input, the three-dimensional (3D) structure of this enzyme was generated using the sophisticated homology modeling tool, namely SWISS-MODEL (Bordoli et al. 2009). Among the various templates recognized by the search algorithm, the best template was chosen, taking various aspects like sequence identity, sequence coverage and best resolution into consideration. 2Z8X, the PDB ID of the extracellular enzyme (lipase), from *Pseudomonas* sp. MIS38 was used as template for model building (Angkawidjaja et al. 2007). The model quality was tested using various methods, including PROCHECK, VERIFY3D, PROSA and by estimating RMSD between model and template (Laskowski et al. 1993; Lüthy et al. 1992; Wiederstein and Sippl 2007).

Preparing protein (lipase) and substrate (tributyrin and oleic acid) coordinates

As a rule of thumb, one has to perform protein preparation before molecular docking. The needful was done by taking advantage of the protein preparation wizard, the component of the Schrödinger suite. During the preparation of protein, the standard protocol was followed (Madhavi Sastry et al. 2013; Mir et al. 2020). After fixing missing side chains and other parameters, the protein was optimized and then refined. The active site was selected by specifying the residues that were confirmed to be active site residues by various tools, including ScanProsite and Computed Atlas of Surface Topography of proteins (CASTp) (De Castro et al. 2006; Tian et al. 2018). Coordinates of tributyrin and oleic acid were retrieved from the huge database, PubChem. The PubChem CID of these molecules is 6050 and 445,639, respectively (Kim et al. 2020). Tributyrin and oleic acid structures were prepared for molecular docking with the help of LigPrep software (LigPrep, Schrödinger, LLC, New York, 2021). Substrates, as usual, were minimized, desalted, metal-binding states and tautomers were generated and default parameter for chirality was opted (Shankaran et al. 2016).

Flexible molecular docking and binding free energy quantification

The prepared tributyrin and oleic acid were docked into the predefined active site of cold-active lipase. As it is well proven that flexible docking in extra-precision mode has better accuracy than standard-precision mode, thus we executed molecular docking in the former mode. Docking was executed using the pandemically authenticated Glide tool. The best pose, in either case, was picked based on docking score criteria (Friesner et al. 2006; Ganai 2021). The individual tributyrin–lipase and oleic acid–lipase docked complexes were used as input for estimating the values of binding free energy. Calculations were done with the popular implicit solvation reliant MMGBSA method. MMGBSA of Prime module performs multiple energy estimations and from those, the binding free energy value is finally deduced using the standard equation;

E_complex (minimized) – E_ligand (minimized) – E_ receptor (minimized) = ΔG (bind).

Default parameters, including the novel energy model, namely VSGB 2.0 were maintained during the calculations in both cases (Ganai 2021; Li et al. 2011).

Results

Identification of psychrotrophic bacterium

The taxonomical study conducted on the lipase-producing psychrotrophic bacterial strain, CRBC14 showed that the strain was rod-shaped, Gram-negative and aerobic with an optimum growth temperature of 20 °C. Besides, the isolate could grow at temperatures between 4 and 30 °C, but could not grow at 37 °C. This behavior towards temperature indicates that it is a psychrotrophic bacterium. The CRBC14 strain was closely associated (100–98%) with *Pseudomonas* sp. based on the 16S rRNA gene sequence review. A high similarity rate was observed with *Pseudomonas* sp. ICMP 13 603 (Fig. 1) after the nucleotide sequence was BLAST searched at NCBI. The sequence of 16S rRNA gene was deposited in NCBI GenBank under the accession number MT478141.

Screening for lipase activity

The lipolytic activity was seen at temperatures between 4 and 30 °C on 1% (v/v) tributyrin, with a maximum at 20 °C and pH 8.0 in comparison to olive oil (Fig. S1). Lipase

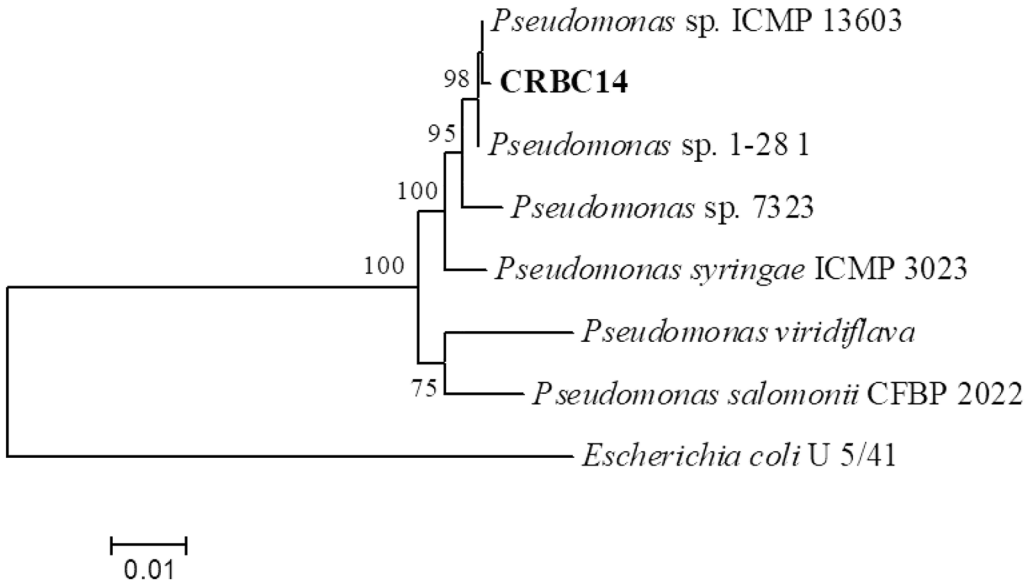

Fig. 1 Phylogenetic tree of strain CRBC14 (indicated by bold letters) based on the sequence of 16S rRNA genes. Using MEGA 7.0 software, the phylogenetic tree was developed from the neighbor-joining approach, and 1000 bootstrap analysis trials were performed

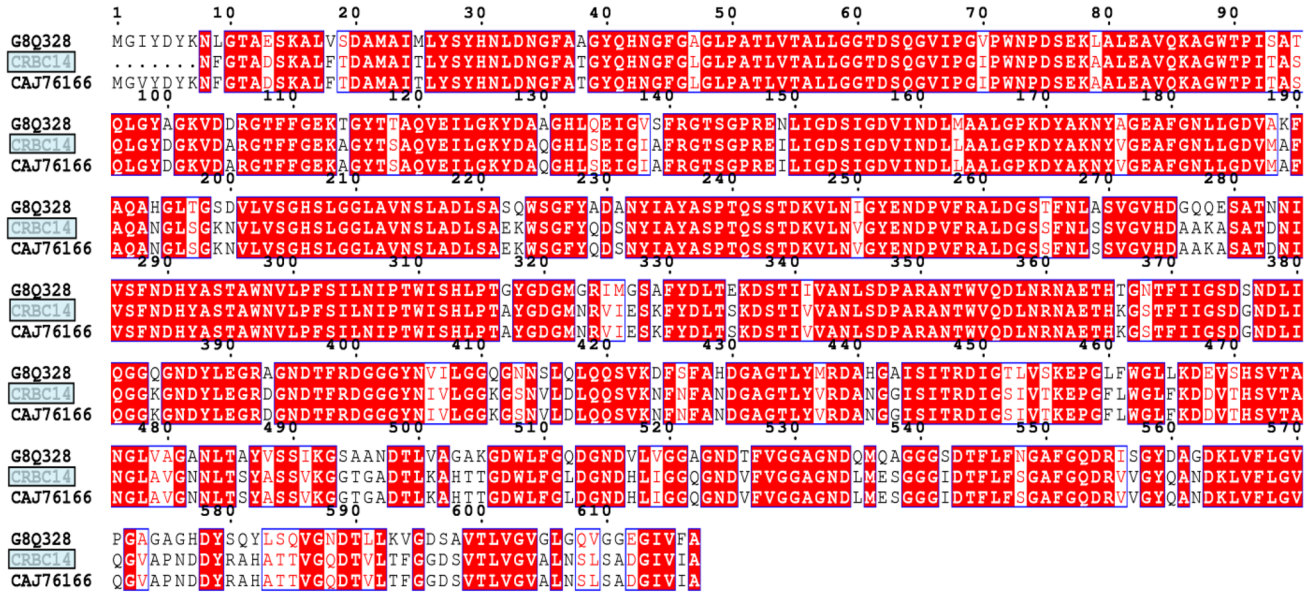

Fig. 2 Similarity analysis of multiple amino acids sequence. The red backdrop highlights exclusively conserved residues, and boxed are the conservatively replaced residues. The vertical row displays the signal peptide's cleavage site, and the horizontal rows show the N-terminal residues

activity increased from 4 to 20 °C, after which the enzymatic activity started decreasing, indicating its cold-active nature.

Amplification of lipase gene and sequence analysis

Sequence analysis of our lipase (GenBank accession no. MW417497) revealed an open reading frame of 1,835 nucleotides with 46% G + C content. The polypeptide sequence of the cold-active lipase showed 99% identity with that of *Pseudomonas* sp. 7323 lipase (GenBank: CAJ76166), 79% with LipA of *Pseudomonas fluorescens* F113 (GenBank: G8Q328) (Fig. 2). The nucleotide sequence encoded a protein comprising 611 amino acids with a calculated molecular mass of 63.71 kDa.

Template guided modeling and validation

The extracellular lipase from *Pseudomonas* sp. MIS38 (PDB ID: 2Z8X) was utilized as template to build the 3D structure of the cold-active lipase. This refined model (red) on superimposing with template (yellow) showed a very low RMSD (0.055 Å), suggesting its structural closeness with the latter (Fig. 3). Additionally, this model qualified stereochemical quality check as its Ramachandran plot showed localization of 92.5% residues in the most preferred region (acceptable quality), 6.5% in the allowed region and 0.2% residues in the disallowed region (Fig. S2). Besides, the structure-sequence compatibility of this model was found to be highly acceptable as the percentage of residues scoring over or equal to 0.2 was found to be 98.52%, the threshold being 80% (Fig. 4). PROSA analysis also supported the acceptable model quality as its z-score aligned with similar-sized proteins possessing experimentally determined structures (Fig. 5).

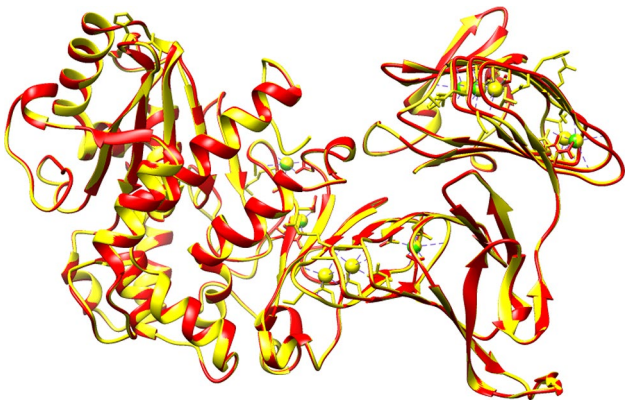

Fig. 3 RMSD between the target (model) and template structure. On superimposing template and target RMSD value of 0.055 Å was estimated. This indicates that the target is very close to the experimentally solved template. For easy understanding, model has been shown in red and the template in yellow

Flexible molecular docking and binding free energy estimation

It was observed from the binding free energy estimates for two docked complexes that tributyrin has a higher affinity for cold-active lipase than oleic acid. While oleic acid's value was estimated to be -29.3826 kcal/mol, tributyrin's value was found to be -33.3136 kcal/mol (Figs. 6 and 7). From the binding free energy estimations on two docked complexes, it can be inferred that tributyrin has more affinity towards cold-active lipase when compared to oleic acid. This crux is taken as tributyrin manifested more negative binding free energy value in comparison to oleic acid. While this value for oleic acid was found to be – 29.3826 kcal/mol, a value of – 33.3136 kcal/mol was demonstrated by tributyrin (Figs. 6 and 7). More negative binding free energy infers more binding inclination.

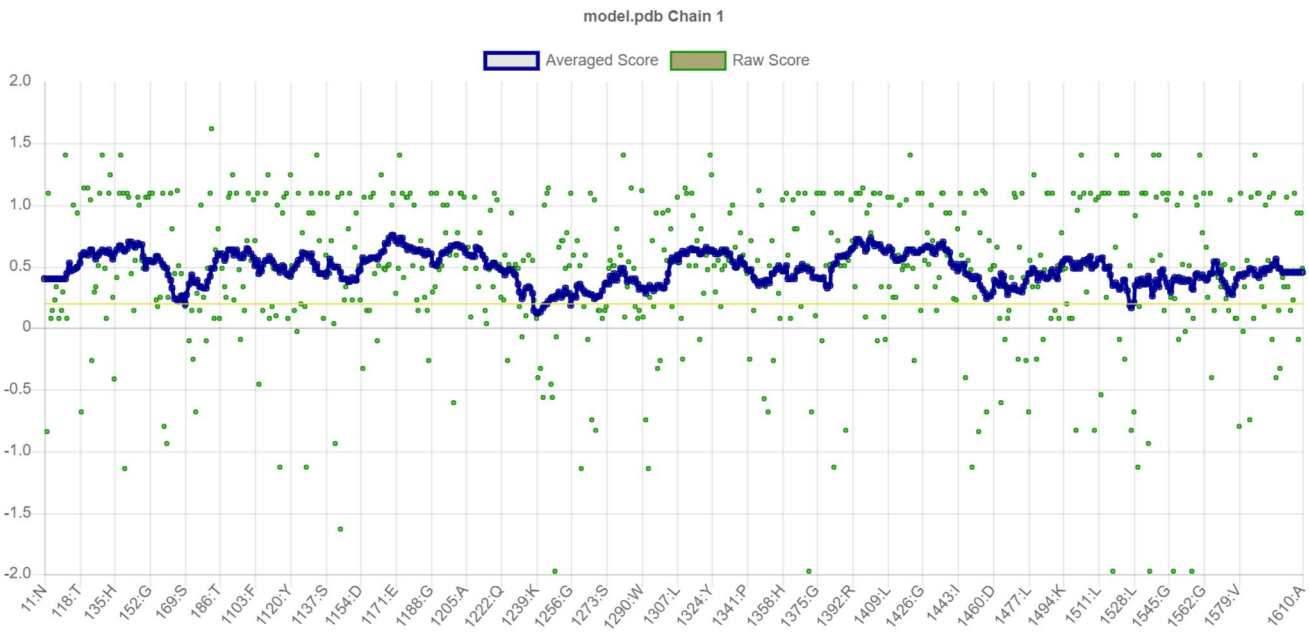

Fig. 4 Measurement of model compatibility with its primary structure (sequence). 98.52% of residues scored beyond or equal to 0.2 (80% lowest limit), thereby confirming the model exactitude

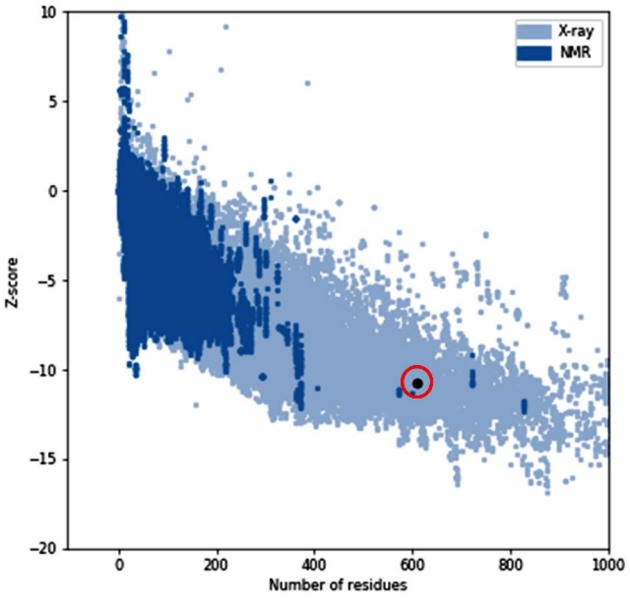

Fig. 5 Testing model quality by estimating z-score. The cold-active lipase model showed z-score in agreement with the z-score displayed by similar-sized proteins whose structures have been solved by empirical methods. The cold lipase model demonstrated a z-score of − 10.7 that falls well within the range

Discussion

Most of the Himalayan glaciers of north-western side are still untouched for their bacterial diversity, which can be a goldmine of potential bacterial enzymes. Isolation of such microbes for the screening and characterization of cold-adapted lipases can bring new opportunities in the enzyme industry. In this study, a cold-active lipase was characterized from a psychrotrophic *Pseudomonas* sp. CRBC14 from a north-western Himalayan glacial soil. The 16S rRNA gene was used for the identification of the isolated bacterium, which is most widely used universal gene marker for psychrophilic and psychrotrophic bacterial identification (Farooq et al. 2021; Rafiq et al. 2017).

Tributyrin has previously been used to determine the lipase activity in psychrophilic and psychrotrophic *Pseudomonas* sp. by analyzing the clear zone around the bacterial colonies grown on tributyrin agar (Maharana and Ray 2015; Salwoom et al. 2019). Bacterial lipases are mostly active in alkaline conditions (Gupta et al. 2004). We also observed the maximum lipase activity (zone of hydrolysis) at pH 8.0 on tributyrin agar. Similar observations were made in cold-active lipases from *Pseudomonas* sp. LSK25 (Salwoom et al. 2019), *Pseudomonas* sp. AKM-LS (Maharana and Ray 2015), *Pseudomonas* sp. KB700A (Rashid et al. 2020), *Pseudomonas antartica* and *Pseudomonas meridian* (Reddy et al. 2004). Furthermore, *Pseudomonas* sp. CRBC14 displayed its optimum lipase activity at 20 °C. This temperature was lower than reported in *Pseudomonas* sp. 7323 (Zhang et al. 2008), *Pseudoalteromonas* sp. 643A (Cieśliński et al. 2007), *Pseudoalteromonas* sp. NJ 70 (Wang et al. 2012), *Pseudoalteromonas Haloplanktis* TAC125, CR9 (De Pascale et al. 2008) where optimal activity was observed at 30, 35 and 40 °C, respectively. While as, cold-active lipases previously isolated from psychrophilic/psychrotrophic *Pseudomonas fragi* X14033 (Alquati et al. 2002), *Pseudomonas* sp. KB700A (Rashid et al. 2020) and *Pseudomonas* sp. 7323 (Zhang et al. 2013) showed optimal activity at 20 °C. Besides, some lipases were reported to show better catalytic activity at low temperatures. For instance, Guo et al (2021) reported a cold-active lipase from *Pseudomonas marinensis* with optimal activity at 4 °C and pH 8.0. In another study, a novel cold-adapted lipase (LipI.3_KE38) from *Pseudomonas fluorescence* KE38 manifested optimal activity at 25 °C and pH 8.5 (Karakaş and Arslanoğlu, 2020).

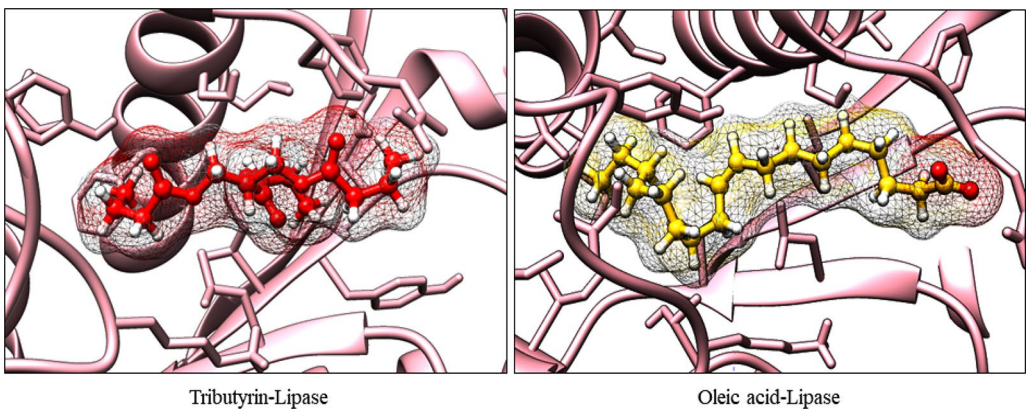

Fig. 6 Tributyrin and oleic acid in the docked state with the lipase. As in both cases, protein is lipase thus, the same color has been used for protein. The ligands being different are presented in two distinct colors. Tributyrin has been shown in red and oleic acid in yellow

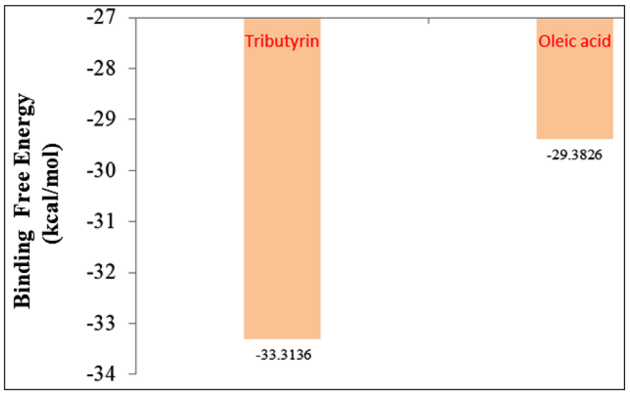

Fig. 7 The binding proclivity of tributyrin and oleic acid towards lipase. From the binding free energy values, it is perceptible that tributyrin–lipase stability is relatively more compared to oleic acid–lipase stability. In other words, tributyrin has higher binding strength with lipase when compared to oleic acid

In silico research has become an important means for discovering and detecting novel enzymes for industrial application (Kwoun Kim et al. 2004). Many analytical tools have been developed to classify conserved genes based on their identical relationships in order to extract the most information from the genome sequences that are currently available (De Pascale et al. 2008). In earlier studies, such approachs have been used to amplify the gene coding cold-active protease and lipase from a psychrophilic *Psychrobacter* sp. 94-6 PB (Perfumo et al. 2020) and *Psychrobacter* sp. G (Xuezheng et al. 2010). The structure of some cold-active lipases from psychrotrophic bacteria has previously been predicted using template-guided modeling methods (Abd. Jalil et al. 2018; Kumar et al. 2020). Moreover, simulation approaches have been used to check the stability of substrates like Triton X-100/toluene with a cold-active lipase from *Pseudomonas* sp. AMS8 (Abd. Jalil et al. 2018). A similar approach was thus used to amplify the gene coding for cold-active lipase from a psychrotrophic bacterium CRBC14 and predict its binding affinity towards two different substrates (tributyrin and oleic acid).

The molecular mass of current cold-active lipase was around 63.71 kDa; Rashid et al (2020) previously reported a similar lipase (CALip) from *Pseudomonas* sp. KB700A with a molecular mass of 49.92 kDa. In addition, psychrotolerant lipases from *Pseudomonas fluorescens* KE38 and *Pseudomonas* sp. AKM-L5 had a molecular mass of 43 kDa and 57 kDa, respectively (Gökbulut and Arslanoğlu 2013; Maharana and Ray 2015). The primary structure of present lipase indicated that it was a member of bacterial lipases (Arpigny and Jaeger 1999). Besides, the amino acid sequence of CRBC14 lipase showed 89% similarity with lipases belonging to group III (subfamily I.3).

The stereochemical quality of model proved to be acceptable as over 92% of the residues were spaced in most favored regions (Mushtaq et al. 2021). While testing the compatibility of primary and 3D structure of model above 98% residues (Fig. 4) scored over or equal to 0.2 further stamping model correctness (Farooq et al. 2021). Apart from this, the z-score of model aligned well with the z-scores of similar length proteins (Fig. 5), having experimentally determined structures suggesting the overall model quality to be highly reliable for docking studies (Wiederstein and Sippl 2007). Moreover, the RMSD was estimated by way of comparing model with a template (Fig. 3). A very low RMSD (0.055 Å) was obtained on comparing these structures, again corroborating model accuracy (Farooq et al. 2021; Pettersen et al. 2004).

On the whole, molecular docking studies coupled with binding free energy estimations revealed that the lipase has more binding inclination towards tributyrin than oleic acid. These results were consistent with our in-vitro studies, where the zone of hydrolysis was found to be more on tributyrin substrate than olive oil (oleic acid). As per the available literature, this was the first approach to use an in silico strategy to characterize the protein sequence of a target gene coding a cold-active lipase from psychrotrophic *Pseudomonas* sp. CRBC14. With a low optimal temperature profile, this lipase could be a potential candidate for industrial uses. Further, investigation of these unexplored Himalayan glaciers could lead to the discovery of new cold-active lipases with unique properties and applications.

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Author contributions SF: data curation, investigation, methodology, writing original draft. SAG: molecular docking, MMGBSA, validation, writing original draft. BAG: supervision, feedbacks, manuscript revision. SM: software license, resources. BU.: formal analysis, visualization. RN: methodology, writing original draft, project administration, resources, supervision.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary file.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval The authors in this study carried out no animal or human studies.

Consent to participate Not applicable.

Consent for publication The publication of this manuscript has been approved by all the authors.

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