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MICROBIOLOGY

Cloning, characterization and functional analysis of lichenase produced by *Bacillus licheniformis* RB16 isolated from cattle faeces

DILEK OZGUN EKIZ, UGUR COMLEKCIOGLU, NAZAN COMLEKCIOGLU & ASHABIL AYGAN

Abstract: Lichenan, 1,3-1,4-β-Glucan, a linear polysaccharide exists in the cell walls of various cereals, has garnered attention for its industrial applications due to its enzymatic breakdown by lichenase enzymes. In this study, *Bacillus licheniformis* strain RB16, isolated from cattle faeces, was identified as a robust lichenase producer. The lichenase gene, *licA*, was successfully cloned and characterized. The cloned RB16 lichenase (LicA) demonstrated its highest activity level at pH 7.5. It also retained over 50% of its activity within the pH range of 6.0–8.5 but experienced a decline to 40% at pH 9.0. LicA was active at temperatures ranging from 25 to 65 °C with an optimum at 45 °C. LicA exhibited more than 60% of its activity at the temperature range of 35–55 °C. Zymogram analysis confirmed LicA's lichenan-degrading ability and structural analysis revealed a stable enzyme structure primarily composed of random coils and extended strands. Although LicA exhibited low thermostability, consistent with its relatively low α-helix content, it demonstrated promising industrial potential. Evolutionary analysis placed LicA within a cluster of closely related *Bacillus* lichenases, particularly *B. halotolerans*, *B. atrophaeus*, and *B. spizizenii*. These findings expand our understanding of lichenases of *Bacillus* and underscore its potential for various industrial applications.

Key words: *Bacillus licheniformis*, cattle, enzyme activity, lichenan, lichenase.

INTRODUCTION

Lichenan, 1,3-1,4-β-Glucan, is one of the essential linear polysaccharides found abundantly in endosperm of rice, barley, sorghum, rye, and wheat. This non starchy polymer consists of β-D-glucosyl units around 1200 and linked to each other through β-1,3 and β-1,4 glycosidic bonds. Hydrolysis of 1,3-1,4-β-Glucan polymer is accomplished by 1,3–1,4- β -D-Glucanases (lichenase, EC 3.2.1.73). The lichenase enzyme has a strict substrate specificity by hydrolyzing the β-1,4 glycosidic bond next to the β-1,3 bond in lichenan molecule while it does not function on β-1,4-glycosidic bonds of carboxymethylcellulose. End product of lichenase enzymes is cellobiosyltriose and cellotriosyltetraose (Chaari et al. 2012). Recently, lichenase enzyme has been tempting the attention of many investigators due to its potentials to be used in brewing, animal feed and detergent industries (Beckmann et al. 2006, Celestino et al. 2006, Mathlouthi et al. 2002). Rather than the lichenase enzymes involved in seed germination in plants those that have been reported as extracellular lichenase from bacteria and fungi have been chosen for bulk production and industrial usage potentials (Ekinci et al. 1997, Huang et al. 2008, Planas 2000).

Among the bacteria, the genus *Bacillus* is the major enzyme source being used in various fields including aquaculture, agriculture, food,

biomedicine, and pharmaceutical industries (Muras et al. 2021). These bacteria found widely in nature, plays a significant role in nutrient cycling owing to its diverse enzyme production; it has been employed in the fermentation industry for more than a decade to produce proteases, amylases, antibiotics, and speciality chemicals, without any reported adverse reaction on human health or the environment, and has been designated as a Generally Regarded As Safe (GRAS) organism (Schallmey et al. 2004).

Lichenases from *Bacillus* are single domain enzymes belong to the family of glycosyl hydrolase 16 (GH16). Considering its beneficial effect, the lichenase enzyme genes from various bacteria like *Bacillus licheniformis* (Teng et al. 2006), *Bacillus halodurans* (Akita et al. 2005), *Bacillus subtilis* (Cantwell & McConnell 1983) along with the fungi *Orpinomyces* (Chen et al. 1997) were cloned and expressed in *Eschericia coli*. In addition to these studies, gene, and protein engineering applications of these enzyme are still of interest for physical and chemical properties preferred for harsh conditions (Olsen et al. 1991, Pavlenko et al. 2019).

Here in this study, we aimed to isolate lichenase producing bacteria from cattle faeces and cloning-expressing into *E. coli*. Characterization of the enzyme along with sequencing and computational analysis of the lichenase gene have also been conducted.

MATERIALS AND METHODS

Isolation and identification

Freshly collected cattle faeces was used for the isolation source. The samples from faeces were diluted using an anaerobic medium. Anaerobic medium was formulated according to McSweeney et al. (2005). Anaerobic medium containing lichenan (0.2%, w/v) as a source of energy to prompt the growth of lichenan-degrading bacteria. Diluted samples were spread onto agar plates consisting of anaerobic medium in an anaerobic cabinet (Elektrotek AW200SG, West Yorkshire, United Kingdom) ensuring strict anaerobic conditions. Then the plates were also incubated at 40 °C under this anaerobic conditions up to 2 days. After incubation period, totally ten lichenan-degrading gram positive bacterial strains were picked and tested for their lichenase-production potentials. Finally, the strain RB16 was chosen for other experiments. The isolated bacterial strain RB16 was identified according to its 16S rRNA sequence analysis. Therefore, the universal primers 27F (5'-AGAGTTTGATCMTGG CTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used for amplification. The isolate RB16 was saved at -80 °C in sterile glycerol stock (60%) for further researches. Evolutionary analyses of 16S rRNA sequences were conducted in MEGA11 (Tamura et al. 2021). The evolutionary distances were determined with the Maximum Composite Likelihood method. The bootstrap consensus tree from 1000 replicates was used to represent the evolutionary history of the taxa analyzed.

Cloning and expression of lichenase gene in *E. coli*

Following the isolation of genomic DNA from RB16 strain, the lichenase encoding gene was amplified using the forward (5'-GGATTGTTTATGAGTTTGT-3') and reverse (5'-TTATTTTTTTGTATAGCGCA-3') primers. To design these primers, the full-length sequences of endo-beta-1,3-1,4-glucanases from *B. subtilis* (Accession numbers U60830 and HQ834722) were obtained from GenBank (http://www.ncbi.nlm.nih.gov). They were specifically designed to cover the entire ORF of the lichenases and were finally synthesized by Ella Biotech GmbH, Germany. The PCR-based amplification of the lichenase gene was accomplished, starting from

the initial denaturation phase at 94°C for 5 minutes, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C. Then the resulting PCR product was identified through agarose gel (1%) electrophoresis. The ligation into the pGEM®-T Easy Vector System I (Promega) was carried out. The plasmid pGEMLA carrying the lichenase insert was introduced into the host *E. coli* DH5α with $CaCl_2$ method (Mandel & Higa 1970) to achieve the expression of the lichenase proteins. After transformation step, *E. coli* cells were cultivated on Luria-Bertani (LB) agar plates supplemented with ampicillin (50 µg/ml). The recombinant cells were examined for lichenase production by overlaying 0.4% agarose in 50 mM sodium phosphate buffer (pH 6.5) including 0.1% lichenin. Lichenase activity was detected by using Congo-red (0.5%, w/v) staining after incubating the plates at 37 °C for 4 hours (Teather & Wood 1982). Excess dye was washed using 1M NaCl, and clear halos around colonies showed the lichenase-positive strains. An *E. coli* strain displaying lichenase activity (designated as *E. coli* L1) was purified and preserved in 20% (v/v) glycerol at -20 °C for further experiments. For lichenase production, *E. coli* L1 was always grown in LB medium supplemented with ampicillin (50 µg/mL) at 37 °C with shaking (150 rpm). After the cells were gathered, they washed with 50 mM sodium-phosphate buffer (pH 6.5) twice. Later on the recovered cells were ruptured with a ball mill (Retsch), and soon after resuspention in 50 mM sodium phosphate buffer (pH 6.5) was performed. The cell debris was separated by a centrifugation step, and finally the resulting supernatant was stored at -20 °C for subsequent lichenase analysis.

Lichenase assays

Lichenase activity was assessed using Miller's dinitrosalicylic acid method (Miller 1959). The quantity of reducing sugar from the enzymatic activity of 0.2% lichenin in 50 mM phosphate buffer (pH 6.5) at 40°C for 30 minutes was measured using a UV-Visible spectrophotometer at 540 nm. Determining one unit of lichenase activity was based on the enzyme's ability to yield 1 µmol of reducing sugar per minute. To investigate the impact of pH on enzymatic activity, the substrate was prepared in different buffer solutions with pH values ranging from 3.5 to 10.0, including 50 mM acetate buffer (pH 3.5 to 5.6), phosphate buffer (pH 6.0 to 8.0), Tris-HCl buffer (pH 8.0 to 9.0), and Glycine-NaOH (pH 9.5-10.0), while maintaining the temperature of 40°C. The optimum temperature for lichenase activity was measured by incubating the reaction mixture at various temperatures ranging from 25 to 65 °C at pH 6.5. Additionally, the pH stability of the lichenase was examined by pre-incubating the enzyme in buffers with different pH from 3.5 to 10.0 for 15 minutes at optimum temperature. On the other hand, the thermostability of the lichenase was tested after pre-incubation of the enzyme at temperatures ranging from 25 to 65 °C for 60 minutes at optimum pH. Following the pre-incubation treatment, the lichenase was chilled for 5 minutes in an ice bath. Subsequently, the residual enzyme activity was measured as defined earlier. Experiments were performed in triplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and zymogram analysis

Enzymatic activity and molecular weight determination were determined using SDS-PAGE electrophoresis (Laemmli 1970). For zymogram analyses, a 12.5% acrylamide-resolving gel containing 0.2% lichenan was prepared. After the solidification of the resolving gel, an appropriate amount of stacking gel (4%) was poured on top of it. The polymerized gel was placed in an electrophoresis tank (Bio-Rad; Mini Protean

Tetra System) containing running buffer (25 mM Tris HCl, 200 mM glycine, 0.1% SDS, pH 8.3). Enzyme samples (10 µl) were mixed with loading buffer and pipetted into the wells. A standard molecular weight ladder was also loaded into the gel for molecular weight estimation. After loading the samples onto the gel, the stacking gel was run at 70 V and the resolving gel at 100 V.

Following the electrophoresis, the gel containing the protein ladder and samples was separated using a sterile scalpel. The gel with the protein ladder was stained with a solution containing 0.1% Coomassie Brilliant Blue R 250, 8% methanol, and 7% glacial acetic acid at room temperature overnight. The excess dye was removed by soaking the gel in a solution containing 8% methanol and 7% glacial acetic acid. The gel containing the enzyme samples was washed with 75% buffer (50 mM phosphate, pH 6.5) / 25% isopropanol (v/v) at 4°C overnight. Subsequently, the gel was taken out of this mixture, washed with 50 mM phosphate buffer (pH 6.5), and then incubated in the same buffer at 37 °C for 4 hours. To determine lichenase activity, the gel was left in 0.1% Congo Red for 30 minutes, and the excess dye was removed with 1 M NaCl.

Detection of end products by thin layer chromatography

Thin-layer chromatography was used to detect the final product or products from the hydrolysis of substrates, depending on the enzyme-substrate reaction (Heftmann 1975). Enzyme reactions were carried out at optimum temperature and pH for 2, 4, and 24 hours. After this stage, to identify the hydrolysis products released, samples and standards (glucose and cellobiose solutions) were spotted onto a silica gel plate (Silica gel 60, Merck, 20x20) in 5 µl volumes. A mobile phase (ethyl acetate-acetic acid-distilled water, 2:2:1 v/v/v) was used for running buffer. The end products were made visible by spraying the plate with 20% H_2SO_4 solutions. The plate was left to dry, and the bands were incubated in a 150 °C oven until they became visible.

Sequencing and computational analysis of the lichenase gene

The cloned lichenase gene was amplified from pGEMLA using PCR. Subsequently, the PCR product was run on a 1% agarose gel and then recovered from the gel using the GF1 AmbiClean Kit following the manufacturer's instructions (Vivantis, Malaysia). The lichenase gene was sequenced on both strands by a commercial company (Macrogen, Korea). Computer programs such as ChromasPro V1.7.7 and Clone Manager 5 were employed to align and analyze the sequence data. The physicochemical properties and amino acid composition of the lichenase polypeptide chain were examined in the ExPASy-ProtParam tool (https://web.expasy.org/protparam/). Protein solubility (Sol) prediction was done using the Protein-Sol tool (https://protein-sol.manchester.ac.uk/). The presence of conserved domains was identified using CDD-BLAST (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). For determining the secondary structure of the enzymes, the SOPMA tool in prabi (https://npsa-prabi.ibcp.fr/) was utilized, which calculated the number of α-helices, turns, extended strands, and random coils. The 3D structure of the lichenase sequence was predicted using SWISS-Model (https://swissmodel.expasy.org/). Additionally, the 3D model of lichenase was evaluated using ERRAT, Verify 3D, and PROCHECK through the SAVES v6.0 web-server (https://saves.mbi.ucla.edu/).

Evolutionary analysis of LicA

Sequence data of lichenases (GH16) were retrieved from the NCBI (National Center for

Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/), and these sequences were utilized to assess the phylogenetic relationships of the lichenase studied in this work. The amino acid sequences of 30 proteins containing the GH16 catalytic domain were aligned using the MAFFT web software (Katoh et al. 2017). The evolutionary history was inferred based on the Neighbor-Joining method, and the evolutionary distances were computed using the Poisson correction method. Finally, evolutionary analyses were performed using MEGA 11 (Tamura et al. 2021).

RESULTS AND DISCUSSION

Isolation and identification of the strain RB16

In this study, a lichenase-producing gram-positive bacterium was isolated from cattle faeces. The strain RB16 exhibit the highest lichenase activity out of 10 potential lichenase-positive bacteria during preliminary screening. The strain RB16 showed significant growth between 25–55 °C (data not shown). The strain RB16 was picked for further experiments and used for 16S rRNA gene sequencing and phylogenetic survey. Genomic DNA of the strain RB16 was run on 1.0% agarose gel, and a separate DNA band of high molecular weight was obtained. The isolated genomic DNA was PCR-amplified with the use of primers 27F and 1492R, and approximately a single discrete 1500 bp PCR amplicon was obtained. Subsequently, the purified PCR amplicon was subjected to sequencing and as result, a consensus sequence of 1433 bp was obtained (data not shown). The constructed consensus sequence was applied to nucleotide blast, and the percent identity of the first 5000 matches ranged between 99.65-97.13%. Based on nucleotide BLAST analysis, strain the RB16 was identified as *Bacillus licheniformis* according to their distances the nearest neighbour regarding sequence homology. Hence, the identified strain was designated as *Bacillus licheniformis* strain RB16. The strain RB16 was compared with the sequences of *B. licheniformis*, *B. paralicheniformis* and *B. amyloliquefaciens* with maximum identity and aligned using MAFFT. The phylogenetic tree was also built using MEGA11 software, as indicated in Figure 1.

Cloning and characterization of LicA

The DNA fragment amplified using the lichenase primers designed from *B. subtilis* resulted a length of 650 bp. It was then inserted into the pGEM®-T Easy Vector, resulting in the creation of a new vector called pGEMLA. The pGEMLA vector was introduced into *E. coli* DH5α. Since *E. coli* DH5α did not naturally produce lichenase, only colonies with a successful insertion of the *licA* gene showed lichenase activity. One such desired recombinant colony was isolated and designated as *E. coli* L1 as a result of the transformation process. To confirm the existence of the *licA* in the recombinant plasmid (pGEMLA), PCR amplification was performed using the same lichenase primer pair. The complete nucleotide sequence of *licA* was obtained from pGEMLA and the length of the sequenced *licA* was 655 bp with a GC content of 42.9%. *licA* had a open reading frame (ORF) with a length of 543 bp. This ORF encoded a polypeptide consisting of 181 amino acids. The predicted molecular mass (MW) and isoelectric point (pI) of the polypeptide were calculated to be 20.490 Da and 5.25, respectively.

With an instability index of 15.62, LicA was classified as a stable protein. Furthermore, it displayed an aliphatic index of 63.54 and a hydropathicity average value of -0.435. The proteins with a high aliphatic index value tend to be thermostable (Ikai 1980). Additionally, the lower range of GRAVY (Grand Average of Hydropathicity) suggests that LicA may have

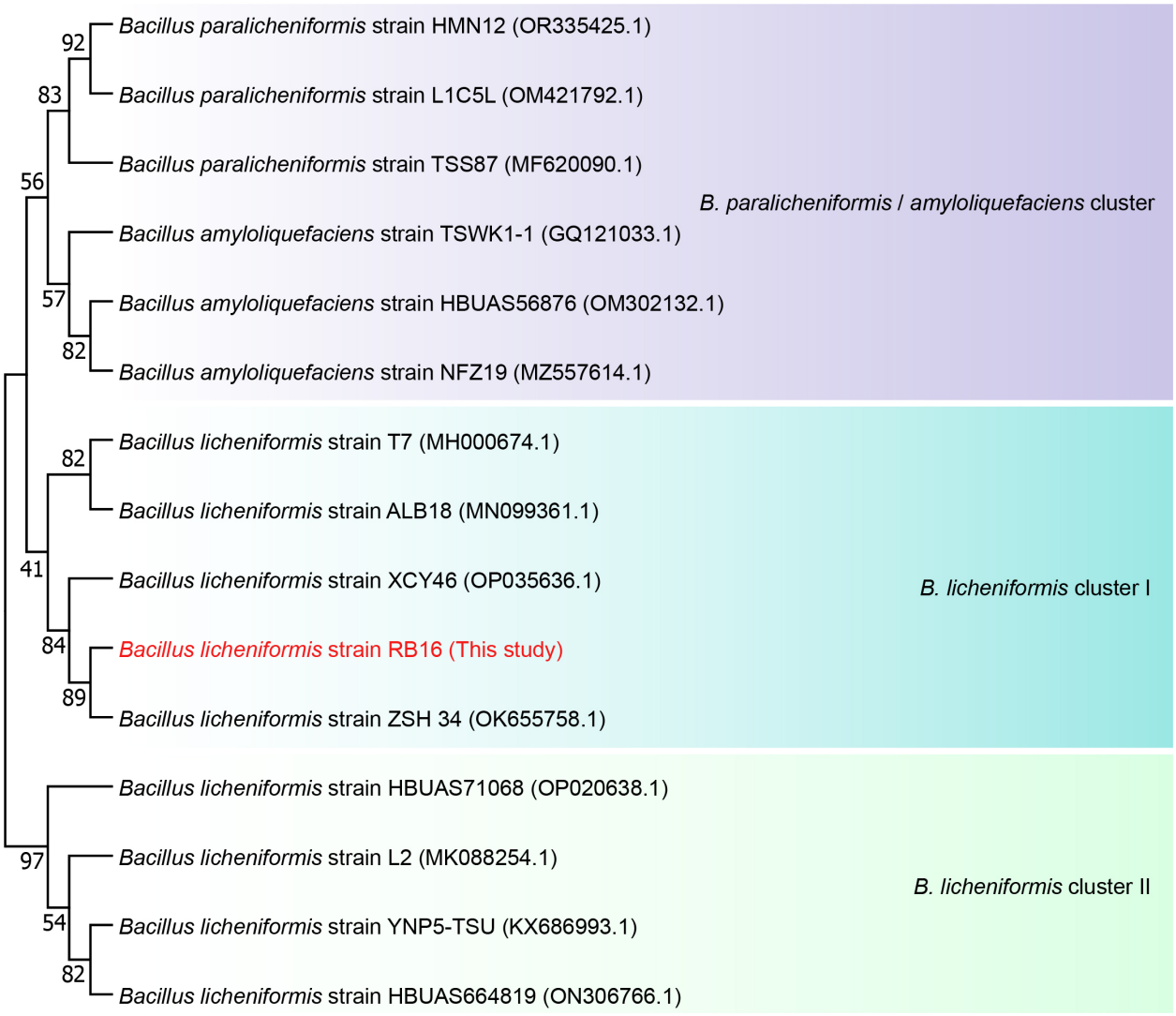

Figure 1. Phylogenetic tree showing the position of *B. licheniformis* strain RB16 used in this study with respect to 16S rRNA sequences. Accession numbers of the closest sequences retrieved from NCBI were given in the tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is indicated next to the branches (Felsenstein 1985). The evolutionary distances were estimated with the Maximum Composite Likelihood method and they are displayed in the units of the number of base substitutions per site. This analysis conducted to sequences of 15 nucleotides. All unclear positions were extracted for each sequence pair (pairwise deletion option). Total of 1546 position were included in the final dataset. Consequently, the evolutionary analyses were performed in MEGA11 (Tamura et al. 2021).

improved water interaction properties (Gupta et al. 2012). The predicted solubility scale of LicA was determined to be 0.56, surpassing the threshold level of 0.45, which indicates that LicA is soluble. Estimating the solubility of proteins intended for recombinant production is crucial as it helps avoid additional costs by eliminating insoluble proteins from the experimental trials (Ghomi et al. 2020).

The main amino acid residues found in LicA were Thr (9.4%), Gly (9.4%), Asn (7.7%), Tyr (7.2%), Val (6.6%), and Asp (6.1%). LicA contains a high volume of glycine and a low portion of proline (4.4%) and arginine (2.2%), which indicates a

likelihood of a flexible structure for lichenase being studied. A reduced rate of arginine and proline in an enzyme has been associated with increased structural flexibility (Latip et al. 2019). On the other hand, a higher percentage of glycine (9.4%) is known to serve as a flexible link in proteins, contributing to their flexibility (Hall 1998).

Several *Bacillus* species producing lichenases, along with the genes encoding these enzymes have been subjected to cloning and characterization, including *B. licheniformis* (Chaari et al. 2012), *B. subtilis* (Qiao et al. 2009), *B. amyloliquefaciens* (Wang et al. 2021), *B. brevis* (Louw et al. 1993), *B. polymyxa* (Gosalbes et al. 1991), *B. circulans* (Bueno et al. 1990). Almost all 1,3-1,4-β-glucanases from *Bacillus* exhibit similarity in sequences of both amino acid and nucleotide composition. The conserved sequences of LicA were evaluated with the use of the InterPro and NCBI's Conserved Domain Search tools. LicA harboured the conserved motif EIDIEF (Figure 2) of glycoside hydrolase family 16 located from amino acids 77 to 82, hence analysis revealed that LicA contains the catalytic domain of the GH16 family.

Substrate specificity of LicA

To assess the specificity of LicA from *B. licheniformis* RB16 towards glycosidic bonds of lichenan, laminarin, β-glucan, CMC along with xylan were tested. Lichenan contains both β-1,3 and β-1,4 glycosidic bonds, while laminarin and cellulose have β-1,3 and β-1,4 glycosidic bonds, respectively. On the other hand, xylan contain also β-1,4 glycosidic bonds of xylose residues, so bifunctional enzymes having xylanase and lichenase activities were reported that act on both lichenan and xylan (Ekinci & Flint 2001, Shi et al. 2010). As a result of the investigation, the activity of LicA for lichenan (61.21 U/mg) was found to be much higher than that for β-glucan (15.91 U/mg). LicA demonstrated no activity on laminarin, CMC, or xylan (Table I). Therefore, it is concluded that LicA can only break down the β-1,4-glycosidic bonds next to β-1,3-glycosidic bonds. Lichenase from *B. licheniformis* GZ-2 had more activity on lichenan than β-glucan (Gao 2016), while the hydrolysis activity of lichenase from *B. subtilis* 168 (Furtado et al. 2011) and *B. licheniformis* EGW039 (Teng et al. 2006) on barley β-glucan was higher than on lichenan.

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Strain RB16 FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVDLGFDAANAYHTYAFDW
WP_2859774  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVDLGFDAANAYHTYAFDW
OWV38346.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVDLGFDAANAYHTYAFDW
WP_2121105  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVDLGFDAANAYHTYAFDW
WJE44936.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVDLGFDAANANHTYAFDW
AAQ88441.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKLVNLGFDASNSYHTYAFDW
AAQ67340.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKLVDLGFDASNSYHTYAFDW
ACX42225.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKLVDLGFDASNAYHTYAFDW
CBJ54979.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKLVDLGFDASNSYHTYAFDW
AAN64132.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKVADLGFDAANAYHTYAFDW
AEK21297.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKVADLGFDAANAYHTYAFDW
ACC77699.1  FTYTGPTEGTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKLADLGFDAANAYHTYAFDW
AFX61538.1  FTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVNLGFDAANSYHTYAFDW
            *******:****************************.*****: :*****:*: *******
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Figure 2. Homology alignment of lichenases from *B. licheniformis* (AAQ88441, AAQ67340, CBJ54979, ACX42225, AAN64132), *B. atrophaeus* (WP_285977488), *B. intestinalis* (OWV38346), *B. paralicheniformis* (WP_212110595), *B. halotolerans* (WJE44936), *B. subtilis* (AEK21297), *B. amyloliquefaciens* (ACC77699), *B. altitudinis* (AFX61538). The conserved motif of GH16 was shown shadowed.

Effects of pH and temperature on LicA activity

The cloned RB16 lichenase (LicA) demonstrated its highest level of activity at pH 7.5. The remaining activity was over 50% within the pH range of 6.0–8.5 but a decline to 40% at pH 9.0 was experienced, as depicted in the Figure 3b. The optimal pH for LicA activity was comparable to that of other *B. licheniformis* lichenases, which typically show their peak activity around pH 6.0–7.5 (Chaari et al. 2012, Gao 2016). However, it differed from enzymes found in other *Bacillus* species such as *B. brevis* (optimal pH 9.0) and alkalophilic *Bacillus* sp. N137 (maintaining over 80% activity in the pH range of 7.0–12.0) (Planas 2000). The pH stability of LicA was studied at various pH values (3.5–10.0) with the incubation of the enzyme in the absence of the substrate at optimum temperature for 1 h. Finally, the

Table I. Substrate specificity of cloned LicA.

Substrate	Specific activity (U mg^{-1}) ± SE[a]	Total activity (U ml^{-1}) ± SE[a]
Lichenan	61.21 ± 1.58	10.28 ± 0.14
Barley β-glucan	15.91 ± 2.28	2.20 ± 0.30
Laminarin	ND[b]	ND[b]
CMC	ND[b]	ND[b]
Xylan	ND[b]	ND[b]

[a] Standard error.
[b] Not detectable.

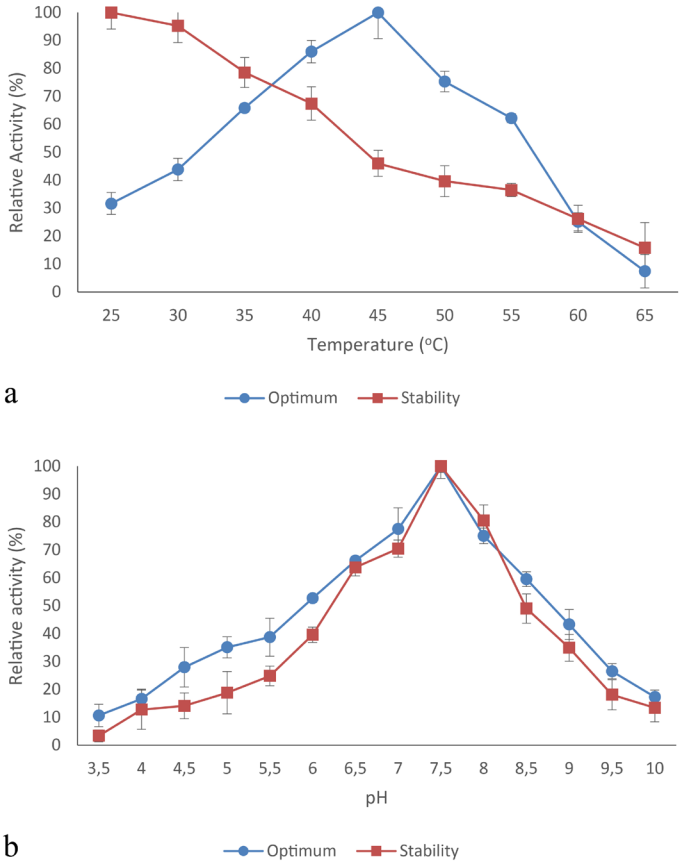

Figure 3. (a) Optimum temperature and thermostability of LicA. (b) Optimum pH and pH stability of LicA.

remaining activity was tested under standard assay conditions. LicA maintained more than 50% of its original activity between pH 6.0 and 8.0. However, LicA lost more than 80% of its activity at pH 4.5 and 9.5. Unlike this study, two lichenases from *B. licheniformis* UEB CF were found to be stable from pH 7.0 to pH 10.0 after 1h preincubation at 40 °C (Chaari et al. 2012). Moreover, lichenase from *Bacillus* sp. UEB-S retained its activity at pH ranging from pH 7.0 to 11.0 for 48 h at 4 °C (Maktouf et al. 2015). The difference in pH stability between this study and other alkaliphilic *Bacillus* spp. may result of the environment where the bacteria live. Endoglucanase, β-glucosidase and xylanase of *B. licheniformis* JK7 studied from the rumen of a native Korean goat were stable in the pH between 3.0 to 6.0 (Seo et al. 2013), which is similar to this study. The pH of the rumen environment fluctuates between 5.8 to 6.7 (Baek et al. 2022), and the pH optimum and pH stability of the enzymes of rumen microbes are generally consistent with rumen pH (Ekiz et al. 2022, Gao et al. 2022).

As given in Figure 3a, the cloned LicA was active at temperatures ranging from 25 to 65 °C with an optimum at 45 °C. LicA presented more than 60% of its activity at the temperature range of 35–55 °C. The relative activity at 25 and 60 °C were 31% and 25%, respectively. However, the enzyme remained active at 75 °C and presented an activity less than 15% of its original activity. Most *Bacillus* species produce β-1,3-1,4-glucanases, but most of these enzymes have optimal temperatures below 65 °C. For instance, the optimal temperature for the *B. subtilis* MA139 enzyme was found to be 40 °C (Qiao et al. 2009), while the *B. subtilis* 168 lichenase exhibited its best activity at 50 °C (Furtado et al. 2011). Two thermostable lichenases from *B. licheniformis* UEB-CF were purified, and the optimum temperatures of these enzymes were 60 and 70 °C (Chaari et al. 2012). LicA was preincubated at temperatures ranging from 0 to 65 °C for 60 min. The remaining activity decreased with increasing temperature. After incubation at the optimum temperature (45 °C) of LicA for 60 min, LicA lost 42% of activity. The thermostability of lichenases produced by *B. subtilis* GN156 and *B. licheniformis* UEB-CF were higher than LicA (Apiraksakorn et al. 2008, Chaari et al. 2012).

Hydrolysis products

Lichenan degradation using the crude LicA preparation was monitored at intervals of 0, 2, 4, and 24 hours. The migration of the degradation product observed through TLC (depicted in Figure 4a) occurred at a position lower than that of cellobiose. The primary products became noticeable even in the initial stages of the reaction, and their concentration increased as the reaction proceeded over time. It is established that endo-β-1,3–1,4-glucanases (lichenases) cleave β-1,4 linkages adjacent to β-1,3 bonds within glucans, resulting mainly in trisaccharide (3-O-β-cellobiosyl-d-glucopyranose) and tetrasaccharide (3-O-β-cellotriosyl-d-glucopyranose) formations (Chaari et al. 2012, Hong et al. 2009, Yang et al. 2008).

Zymogram analysis

The homogeneity of LicA was assessed using SDS–PAGE, which was subsequently followed by zymogram staining. The activity staining of the LicA demonstrated a single band that corresponded to a distinct zone of lichenan breakdown. The cloned enzyme LicA presented a distinct molecular weight, 26 kDa (Figure 4b). This is so close to that of *Bacillus licheniformis* UEB CF (30kDa) (Chaari et al. 2012) and *Bacillus brevis* (29kDa) (Louw et al. 1993) lichenases. In Fig. 4b, the enzyme activity staining demonstrated a single band approving

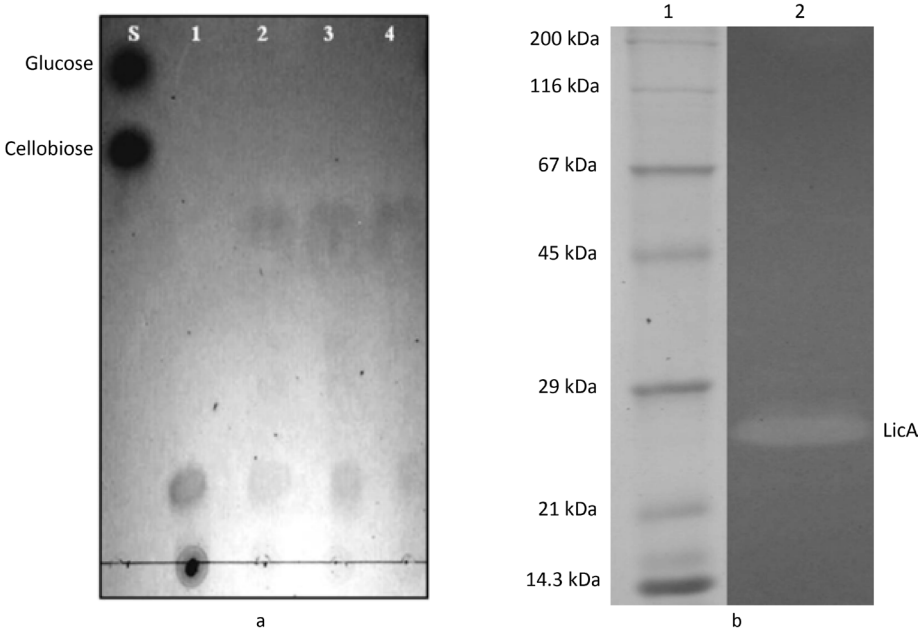

Figure 4. (a) TLCs of hydrolysis products from lichenan produced by LicA from *cloned E. coli* L1 at 40 °C and pH 6.5. S: 1 mg/ml standard (Merck, Germany), Lane 1: 0 min incubation, Lane 2: 2 h incubation, Lane 3: 4 h incubation, Lane 4: 24 h incubation, (b) Enzyme control. licA expression analysis with 12.5% SDS-PAGE containing 0.2% lichenan. Lane 1: Protein molecular weight markers, Lane 2: Cell lysate of *E.coli* L1.

hydrolysis process. About 5.5 kDa difference between the *in silico* MW estimation and the zymogram analysis estimation was assumed to be due to electrophoresis conditions.

Structural analyses of LicA

The secondary structure of LicA was primarily composed of random coils (50.83%) and extended strands (37.02%), followed by β-turns (6.63%) and α-helix (5.52%). Increased α-helix content provides proteins with enhanced stability at elevated temperatures, whereas thermophilic proteins tend to have lower amounts of sheet regions due to their susceptibility to heat (Kumar et al. 2014). In this context, low thermostability of LicA was consistent with the smaller proportion of α-helix.

The tertiary structure of LicA was modelled based on homology with beta-1,3-1,4 glucanase from *Bacillus subtilis* strain 168 (PDBe: 3o5s) and evaluated using the Swiss-Model workspace's structural assessment server. The sequence identity between LicA and *B. subtilis* enzyme was 96.13%, with a coverage of 99%. LicA existed as a monomeric oligostate, and its GMQE and QMEAN values were 0.83 and 0.79, respectively. A higher GMQE score indicates greater reliability (Biasini et al. 2014), while QMEAN scores of -4.0 or below suggests low model quality (Benkert et al. 2011).

Examination of the Ramachandran plot revealed that 97.77% of LicA residues were within the ideal region. A quality model is supposed to have over 90% of residues in the favoured region (Pramanik et al. 2017). LicA's protein model achieved an overall quality factor of 93.06%, according to ERRAT. Verify 3D analysis indicated that 88.40% of residues in LicA had an average 3D-1D score of ≥ 0.1. PROCHECK analysis demonstrated that a substantial 100% of LicA enzyme residues resided in favoured and allowed regions, indicating a reasonable level of stereochemical accuracy in the constructed model. The combined findings from ERRAT, Verify 3D, and PROCHECK web tools collectively confirm the excellent 3D protein model quality of LicA from *B. licheniformis* RB16.

The LicA's catalytic site contains a negatively charged crevice encircled by several aromatic unites (Figure 5a). Superposition of the *B. licheniformis* RB16 LicA structure with *B. subtilis*

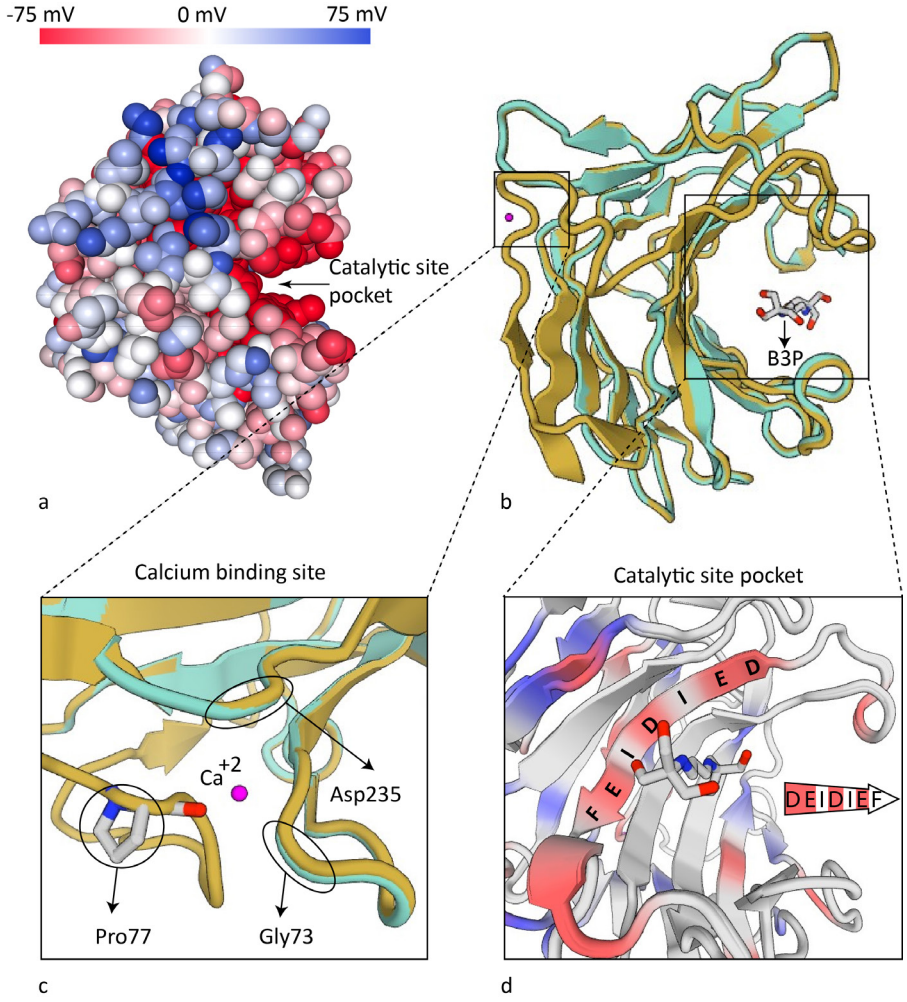

Figure 5. (a) Protein-sol visualization of surface patches on the LicA of *B. licheniformis* RB16. The enzyme was color-coded from negative charge (red) to positive charge (blue). LicA was visualized with the embedded NGL viewer on the protein-sol web application. Negative charged residues dominated the catalytic site pocket of LicA. (b) Superposition of the LicA structure with the crystal structure of endo-beta-1,3-1,4 glucanase from *B. subtilis* 168 (PDBe: 3o5s). Green color indicates LicA and yellow color indicates lichenase of *B. subtilis* 168. B3P (2-[3-(2-Hydroxy-1,1-Dihydroxymethyl-Ethylamino)-Propylamino]-2-Hydroxymethyl-Propane-1,3-Diol) shown in the catalytic site had been used as a ligand in endo-beta-1,3-1,4 glucanase from *B. subtilis* 168. (c) Detailed image of the calcium-binding site of superposed enzymes. Residues coordinating the calcium ion were shown in circles. The positions of the residues were given according to the lichenase of *B. subtilis* 168. Both enzymes covered Asp and Gly, while Pro was present only in the lichenase of *B. subtilis* 168. (d) Detailed image of the catalytic site of the superposed enzymes. Red and blue colors indicated the negative and the positive charged residues, respectively. The positions of the conserved catalytic residues (DEIDIEF) were depicted in the image.

orthologue reveals a similar three-dimensional arrangement (Figure 5b). LicA sequence displayed seven differences compared to the template. Met69, Ala83, Thr113, Ala155, Asn198, Thr215 and Asn228 in the lichenase of *B. subtilis* 168 had replaced with Val, Ser, Ala, Val, Ser, Ile and Thr in LicA, respectively (Furtado et al. 2011). Similar to this study, a lichenase from *Bacillus* sp. UEB-S (Maktouf et al. 2015) had the amino acids of Val and Ser instead of Met69 and Ala83 in the lichenase of *B. subtilis* 168. Glu77 and Glu81 were found in the catalytic site of LicA as the nucleophile and the acid catalyst, respectively (Figure 5d). *B. subtilis* 168 (Furtado et al. 2011)

and *Bacillus* sp. UEB-S (Maktouf et al. 2015) had amino acids of Pro37, Gly73, Asp235 coordinating a single calcium ion on the opposite side of the catalytic site. Gly and Asp residues were present in the LicA but Pro was not found in the cloned LicA since the primers did not cover this residue (Figure 5c). Welfle et al. (1995) proposed that the arrangement of the calcium ion binding site could account for the varying degrees of stabilization observed in three hybrid glucanases upon binding with calcium ions. This observation could elucidate why the LicA displays lesser stability in comparison to the lichenases from *B. subtilis* 168 (Furtado et al. 2011) and *Bacillus* sp. UEB-S (Maktouf et al. 2015).

Evolutionary analyses of LicA

A similarity search for the LicA gene sequence of *B. licheniformis* RB16 was performed in the BLAST server, and 1000 aligned sequences were displayed. The distribution of belonging genus of these 1000 hits according to their BLAST maximum score was given in Figure 6a. The percent-identity ranged between 98.90% and 60.22%. The genera of *Bacillus* and *Paenibacillus* constituted 43.3% and 27.5% of these hits, respectively. Also, the *Streptococcus*, *Ruminococcus* and *Clostridium* genera accounted for 7.9%, 4.5% and 4.2% of these matches, respectively.

According to BLAST results, the representatives of each genus that have the highest similarity were selected for the evolutionary analysis. The evolutionary relationships of LicA and other lichenases were resolved with Neighbor-Joining method (Chaari et al. 2012), then a phylogenetic tree was produced (Figure 6b). Phylogenetic analysis was accomplished on the basis of amino acid sequences of the enzymes. Total 40 enzyme amino acid sequences were used for alignment. The evolutionary analysis indicated that, amongst very similar *Bacillus* species, LicA of *B. licheniformis* strain RB16 grouped with the lichenases of *B. halotolerans*, *B. atrophaeus* and *B. spizizenii*. Also, LicA was closely linked with different *Bacillus* species that contained *B. rugosus* and *Bacillus inaquosorum*. Cluster I consisted of two groups. The first one included bacteria belonging to the genus *Bacillus*. In the second group, the lichenases belonging to *Paenibacillus*. *P. tengchongensis* and *P. spiritus*. On the other hand, other *Paenibacillus* species grouped separately. While lichenases belonging to *Clostridium* clustered in one group, *Streptococcus* and *Ruminococcus* included in the same group.

CONCLUSIONS

This investigation yields valuable insights into *Bacillus licheniformis* strain RB16 and its lichenase enzyme, LicA, sourced from cattle faeces. LicA exhibits notable specificity in targeting lichenan, efficiently cleaving β-1,4 glycosidic bonds proximal to β-1,3 bonds, resulting in the generation of cellobiosyltriose and cellotriosyltetraose. Structural analysis uncovers LicA's stable configuration, characterized primarily by random coils and extended strands. Evolutionary analysis places LicA within a cluster of closely related *Bacillus* lichenases. These findings enrich our understanding of LicA's intrinsic properties,

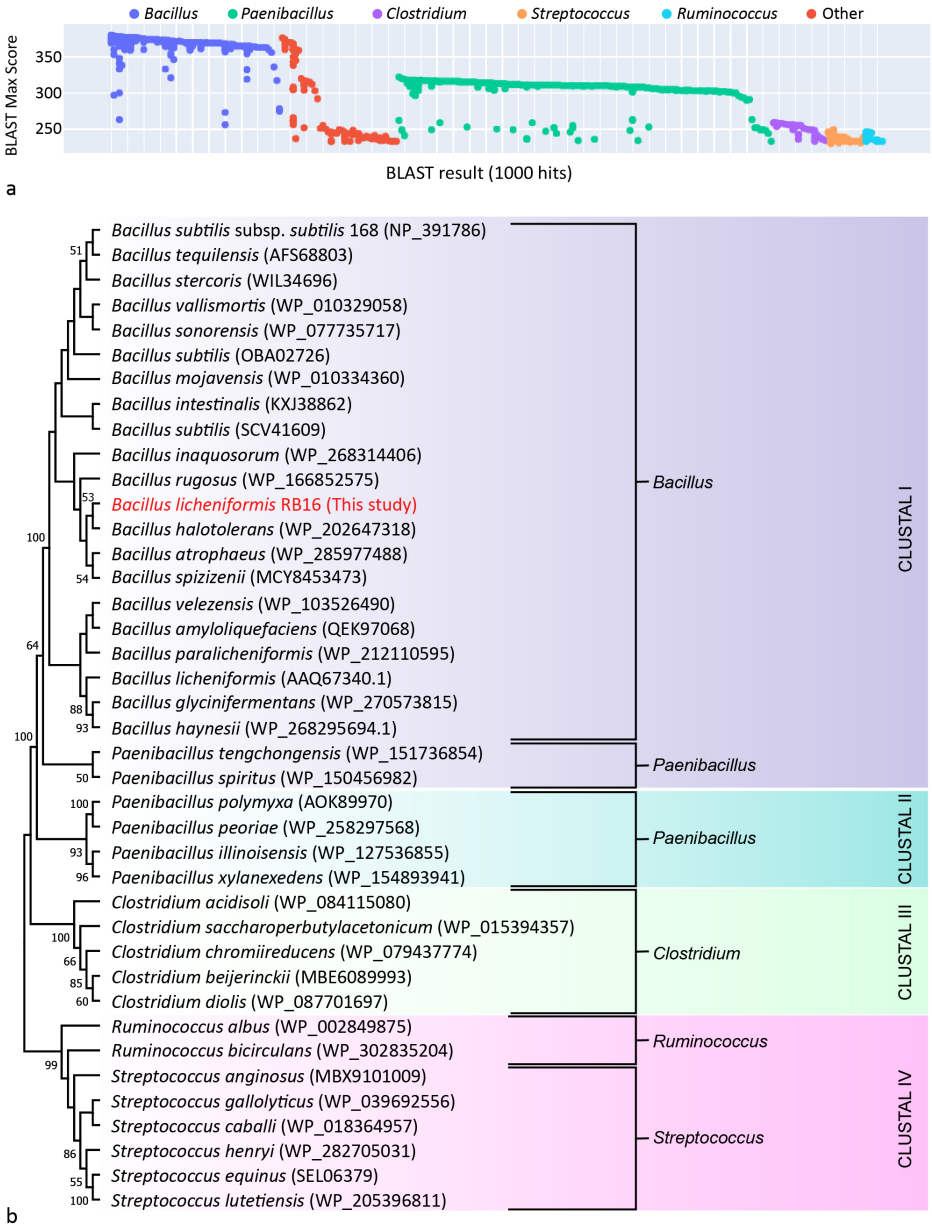

Figure 6. (a) Scatter plot for the BLAST results of LicA. (b)The optimal tree is given. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test(100 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were determined using the Poisson correction method and are in the units of the number of amino acid substitutions per site. This analysis involved 40 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 165 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021). Taxonomic families were given based on the NCBI Taxonomy.

providing a solid foundation for further exploration and research endeavours.

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