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Conserved Protein YpmR of Moderately Halophilic *Bacillus licheniformis* has Hydrolytic Activity on p-Nitrophenyl Laurate

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ABSTRACT

Objective: Hydrolases are of great use in many industries including food, textile, paper, detergent, and pharmaceutical production. These enzymes are abundant in all eukaryotic and prokaryotic organisms. Microbial enzymes are relatively tolerant to changes in pH, temperature and salt concentration and are capable of catalyzing reactions with high substrate specificity. Therefore they are potentially important for industrial applications. In this study we aimed to clone and characterize a hypothetically defined moderately *Bacillus licheniformis* YpmR enzyme, a member of the SGNH-hydrolase superfamily.

Materials and Methods: The hypothetical YpmR gene was amplified with PCR using specific oligonucleotide primers and genomic DNA of *B. licheniformis*. The purified PCR products were cloned under the control of *Escherichia coli* lac promoter. Expression of the recombinant YpmR protein in the *E. coli* cells was assessed using SDS-PAGE/Western blotting. The enzymatic activities were spectrophotometrically determined using p-nitrophenyl laurate (pNPL) and p-nitrophenyl acetate (pNPA).

Results: The YpmR enzyme showed a 7-8 fold higher enzymatic activity against the pNPL substrate as compared to the negative controls. Hydrolysis of the pNPL substrate was found to be due to the *B. licheniformis* YpmR enzyme. In contrast, high hydrolytic activity in bacterial lysates not encoding YpmR enzyme on pNPA substrate indicated that the hydrolysis is due to the presence of other intracellular hydrolases. *B. licheniformis* YpmR enzyme was shown to be tolerant to high NaCl and Triton X-100 concentration.

Conclusion: The moderate halophilic *B. licheniformis* hypothetical YpmR enzyme heterologously synthesized in *E. coli* cells has hydrolytic activity on pNPL substrate. The enzyme was observed to be more tolerant to an increase in NaCl and Triton X-100 concentrations compared to the *Candida rugosa* lipase enzyme used in this study as a control.

Keywords: YpmR gene, Gene cloning, *Bacillus licheniformis*, Hydrolase activity, p-nitrophenyl laurate (pNPL)

INTRODUCTION

Hydrolases are a class of enzymes that catalyze covalent bond cleavage in the presence of water molecules. Some members of this class of enzymes include the carboxylesterases and proteases that act on ester bonds like lipases and esterases and amide bonds in peptides, respectively (1,2). Hydrolases have vast applications in food, detergent and leather industries as well as pharmaceutical industries (3,4). These enzymes are divided into superfamilies based on the type of 3D structural futures and this includes the broader group α/β-hydrolase superfamily with Ser, His and Asp residues as the catalytic triad and the relatively recently identified SGNH-hydrolase superfamily (a diverse family of lipases and esterases) (5,6). A large number of hydrolases in this family have a common catalytic Ser, oxyanion-hole forming Gly, Asn, and invariant His residues (2,7). Different substrates were described for the members of SGNH-hydrolases including acyl-CoA esters (8) lysophospholipids (9), and complex polysaccharides (10). Lipases (EC 3.1.1.3, triacylglycerol acyl hydrolases) are members of the hydrolase

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family that catalyze both the hydrolysis and synthesis of long chain triacylglycerol in the presence or absence of a water medium respectively (11). Lipases are of animal, plants and microbial origins. Among the different sources of lipases, microbial lipases have displayed greater popularity due to their diverse properties and ease of genetic manipulations (11-14). One property of microbial lipases that is of particular importance in many biotechnological transformations is their ability to catalyze reactions in a wide range of temperatures (15,16). As a result, many attempts has been made to isolate new microbial lipases that have the potential to remain active in harsh industrial reaction conditions like high temperatures, presence of alkaline or acidic conditions, etc. The isolation of such robust new enzymes is largely through genetic engineering of the already existing ones or screening for new microorganisms (17). Recently, a new member of the SGNH-hydrolase superfamily called the "SGNH-hydrolase YpmR like" subfamily was identified. This enzyme was reported to have different tertiary fold as compared to the alpha/beta hydrolase family and is generally unique among all the known hydrolase enzymes with an active site similar to the Ser-His-Asp (Glu) triad, but it might lack the carboxylic acid (18,19). Based on sequence alignment analysis, *Bacillus YpmR* gene sequence is reported to be similar to the "SGNH-hydrolase YpmR like" subfamily (19). Despite this finding, the enzymatic activity of the YpmR protein is yet to be experimentally determined. Proteins whose existence has been predicted based on their gene sequences but which lack the experimental evidence of *in vivo* or *in vitro* expression are termed hypothetical proteins. Consequently, the YpmR is only a hypothetical protein with putative hydrolytic activity. Therefore, in a quest to generate novel lypolytic enzymes suitable for both basic research and varied industrial applications, the *Bacillus YpmR* enzyme may be a potential candidate. Hence, the main aim of this study was to seek to establish the hydrolytic activity and enzymatic characteristics of this putative enzyme.

MATERIALS AND METHODS

Chemicals and Reagents

P-nitrophenyl laurate (p-NPL), p-nitrophenyl acetate (pNPA), SDS gel electrophoresis markers and reagents, *Candida rugosa* lipase were purchased from Sigma Aldrich Chemical Co. (St., Louis, USA) and yeast extract and agar from Merck, (Darmstadt, Germany). Bradford reagent and ovalbumin were the commercial product of BioRad (BioRad Laboratories, Hercules CA, USA). All other chemicals were of analytical grade and used without further purification. *Bacillus licheniformis* isolated from nature was kindly provided by Dr. M. Birbir (Biology Department, Marmara University).

Genomic DNA (gDNA) Isolation from Bacteria

Genomic DNA (gDNA) was isolated from the moderately halophilic *B. licheniformis* using a commercial gDNA isolation kit (Invitrogen Cat No. K1820-01). The bacterial cells were cultured in a 5 ml Complex Media at 37°C for 24 hours. The cells were precipitated by centrifuging the culture at 7,500 rpm for 10 minutes. The precipitated cells were used to isolate gDNA based on the manufacturer's protocol.

Construction of Expression Vectors

Hypothetically defined YpmR gene of B. licheniformis was amplified using the forward primer 5'-ATGAACATACGTTTTATTACAGTC-3' (or using 5'-ACATACGTTTTATTACAGTCATG-3' for gene without the ATG start code) and reverse primer 5'-TTATTCTGCTGGGAGGTCTTCG-3'. The primers were phosphorylated using T4 polynucleotide Kinase (New England Biolabs) enzyme prior to their use. All the primers used in this study were originally designed using the NCBI primer design tool. The KOD-plus (Toyobo, Japan) thermostable DNA polymerase enzyme was used for amplification of YpmR gene fragment. The cycling conditions for PCR were: initial denaturation for 3 min at 95°C, 32 cycles of 20 seconds at 95°C, 20 seconds at 55°C and 1 min at 68°C, and in the final extension for 5 min at 68°C. The PCR products were extracted with phenol: chloroform: isoamyl alcohol (25:24:1) followed by ethyl alcohol precipitation. The samples were dissolved in 15 µl of deionized distilled water and purified using agarose gel extraction kit (QIAEX II Gel Extraction Kit). The plasmid vectors pGFP (Clontech) and pET-14b (Novagen) were used for cloning of the YpmR gene. We cloned the amplified *B. licheniformis* YpmR gene with and without histidine (x6 his) tag under the control of *E. coli* lac promoter. The pGFP plasmid vector was linearized using the inverse PCR technique. The vector was amplified using a primer pair targeting the regions bounding the GFP ORF (forward primer: 5'- CATTCGTAGAATTCCAACTG-3' and reverse primer: 5'-AGCTGTTTCCTGTGTGAAATTG-3'). The cycling conditions for this PCR reaction were: initial denaturation for 3 min at 95°C, 32 cycles of 20 seconds at 95°C, 15 seconds at 52°C and 3 min at 68°C, and in the final extension for 10 min at 68°C. The PCR products were purified using gel extraction kit. The PCR products carrying the ATG start codon were ligated with the linearized plasmid vector using T4 DNA ligase enzyme (TaKaRa, Japan). The obtained plasmid vector was named pLac-YpmR. The YpmR gene having a histidine tag coding sequence at 5'-end was cloned in two stages. In the first stage, the gene without ATG start code was cloned into the *Nde*I restriction site of the pET-14b plasmid. For this, the pET-14b plasmid was linearized by cutting it with *Nde*I restriction enzyme (New England Biolabs) and the sticky ends of the plasmid were blunted using the Klenow enzyme (New England Biolabs). For these processes, 5 µg plasmid DNA in 45 µl of deionized distilled water was mixed with 5 µl of x10 concentrated reaction buffer and 1 µl (10U) *Nde*I restriction endonuclease enzyme. The restriction digestion process was carried out at 37°C for 1 hour. Agarose gel electrophoresis was used to check whether the plasmid DNAs were cut. The samples were kept at 65°C for 20 minutes in order to inactivate the restriction enzyme. After the inactivation step, 2.5 µl of 2 mM dNTP mix and 1 µl (1U) Klenow enzyme (New England Biolabs) were added to the reaction mix and incubated at 37°C for 30 minutes. The DNA samples were extracted using phenol: chloroform: isoamyl alcohol and precipitated with ethyl alcohol. The precipitated samples were then dissolved in 22.5 µl deionized distilled water. For the dephosphorylation of plasmid DNA, 2.5 µl of x10 concentrated buffer and 1 µl (1U) of shrimp alkaline phosphatase (SAP; Fermentas) were added to the sample and kept for 1

hour at 37°C and this was followed by incubation of the reaction medium at 65°C for 20 minutes for the inactivation of the SAP enzyme. The plasmid DNA, which was linearized by digestion with *Nde*I restriction enzyme, was ligated with the YpmR gene without the start code (ATG). The plasmid vector obtained was named pET-14b-hYpmR. The hYpmR gene in pET-14b-hYpmR plasmid vectors was sub-cloned under the control of the lac promoter. The pET-14b-hYpmR plasmid was digested with *Nco*I (New England Biolabs) and *Xho*I (New England Biolabs) restriction endonuclease enzymes and the sticky ends were blunted using the Klenow enzyme. The hYpmR DNA fragment was purified with agarose gel extraction kit then ligated with the plasmid DNA obtained from pGFP by inverse PCR as mentioned above. The generated plasmid vector was named pLac-hYpmR.

SDS-PAGE and Western Blotting

The pLac-YpmR and pLac- hYpmR plasmid vectors were transformed in competent *Escherichia coli* Mach1 cells. Small-scale saturated cultures (3 ml) of the transformed bacterial cells were prepared on LB (+amp) medium. The cells in a 0.5 ml samples were precipitated by centrifuging at 7,500 rpm for 5 minutes. The cell precipitates were suspended in 100 µl of deionized distilled water and mixed with an equal volume of 2x concentrated SDS sample loading buffer. The samples were passed through a 27G needle for 10-15 times and centrifuged at 15,000 rpm for 5 minutes. The proteins in the supernatant were denatured at 95°C for 5 minutes and then loaded onto a 10% denatured polyacrylamide gel. Proteins separated by SDS-PAGE were either stained using silver staining technique or transferred to PVDF membranes to detect the hYpmR protein by Western blotting. For blotting, mouse anti-his monoclonal antibody (Santa Cruz # sc-57598) and goat anti-mouse IgG-HRP (Invitrogen # 31420) were used as the primary and secondary antibodies, respectively. The proteins were detected using the ECL Western Blotting Detection Reagent (GE Healthcare # RPN2235).

Preparation of Cell Lysates and Hydrolytic Activity Assays

E. coli cells transformed with the plasmids coding *B. licheniformis* YpmR protein were cultured in 100 ml LB media (+amp) until saturation. Non-transformed *E. coli* cells were cultured in a LB (-). The cells were precipitated by centrifuging at 7,500 rpm for 10 minutes. The cell precipitates were washed with sterile deionized distilled water and then suspended in 5 ml cold Tris-$CaCl_2$ buffer (100 mM Tris-CL, pH: 7; 5 mM $CaCl_2$). Cell suspensions were sonicated on ice with a sonicator (Bandalin) using 80% power 10 seconds on and 10 seconds off for 10 min. The samples were centrifuged at 4°C at a speed of 15,000 rpm for 15 minutes. The Bradford method was used to determine the amounts of protein in the supernatants and these samples were used in the subsequent enzyme activity tests (20). Hydrolysis activities of the cell lysates were determined at different reaction conditions using *p*-nitrophenyl laurate (pNPL) and *p*-nitrophenyl acetate (pNPA). For the enzyme activity assays, 20 µl of substrate (10 mM pNPL or 10 mM pNPA) and 30 µl cell lysate (1-2 µg protein / µl) were added to 950 µl Tris-$CaCl_2$ buffer (pH: 7); followed by incubation of the reaction mixtures at 30°C, 37°C or 55°C for 15-30 minutes.

In order to determine the effects of salt (NaCl) concentration on the hypothetical YpmR enzyme, Tris-$CaCl_2$ buffer containing different concentrations of NaCl (0.1 M, 0.5 M, and 2.5 M final concentrations) were used. In addition, the effect of pH on the hypothetical enzyme was also determined using Tris-$CaCl_2$ buffers adjusted to pH 6 and pH 9.5. To determine the effects of Triton X-100, a non-ionic detergent, different concentrations of the detergent ranging from 0.01% to 1% were added to the reaction mixture. *C. rugosa* lipase enzyme (Sigma-Aldrich # L1754; ≥700 unit/mg solid) solution (15 ng protein/30 µl equivalent to ~ 10 mU) was used as the positive control for the reactions. After incubation, the reaction mixtures were kept at 90°C for 5 minutes in order to inactivate the enzyme followed by centrifugation at 14,000 rpm for 5 minutes. The absorbance (OD_{405}) of samples was determined with a spectrophotometer. The relative enzymatic activities were given as a ratio to the negative control or to the sample showing the lowest enzyme activity.

Statistical Analysis

Statistical methods used in this work include descriptive statistics (arithmetic mean and standard deviation) and the nonparametric Mann-Whitney test. $P < 0.05$ defined statistical significance.

RESULTS

Electrophoretic analysis of recombinant YpmR enzyme produced in *E. coli* cells

The plasmids pLac-YpmR, pLac-hYpmR and pET14b-hYpmR were transformed in *E. coli* Mach1, BL21 and/or JM107 strains, and the YpmR and his-tagged YpmR enzymes produced in these cells were analyzed using SDS-PAGE. The plasmid vectors were found to effectively express in the *E. coli* strains used. Fig-

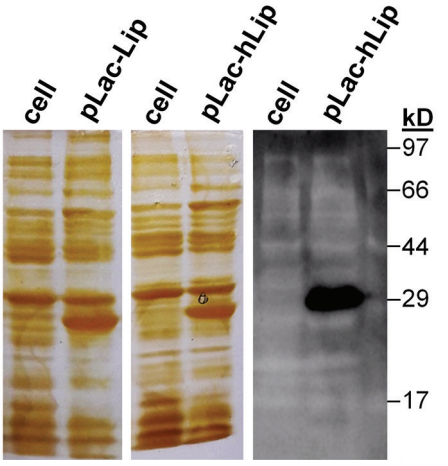

Figure 1. SDS-PAGE/silver staining (left two panels) and Western blotting (right panel) analysis of *B. licheniformis* hypothetical YpmR enzymes synthesized in *E. coli* Mach1 cells transformed with pLac-YpmR and pLac-hYpmR plasmids. The proteins were separated on a 10% denatured polyacrylamide gel. The mouse monoclonal anti-his antibody and HRP-conjugated goat anti-mouse IgG were used as the primary and secondary antibodies for the Western blotting, respectively.

ure 1 shows the SDS-PAGE/silver staining and Western blotting results of the heterologously expressed recombinant *B. licheniformis* YpmR enzymes in *E. coli* Mach1 cells.

Enzymatic Activity of *B. licheniformis* YpmR on pNPL and pNPA

Enzyme activities of the lysates prepared from the transformed *E. coli* cells (*E. coli* BL21 and/or *E. coli* JM107) encoding the *B. licheniformis* hypothetical YpmR enzyme were determined through spectrophotometry using pNPL and pNPA as substrates. The activity of the recombinant YpmR enzyme produced in two different bacterial strains was 7-8 fold higher ($P \leq 0.01$) than that of the non-transformed control cells using pNPL substrate at 37ºC, in Tris-$CaCl_2$ buffer (pH 7). The purified *C. rugosa* lipase enzyme used as the reference in our experiment (Sigma # L1754; 15 ng protein equivalent to ~ 10 mU), produced high level of hydrolysis activity on pNPL substrate (Figure 2A). In contrast, using pNPA substrate, a similar level of enzyme activities (0.9-1.2-fold changes) ($P \geq 0.05$) was detected in both the transformed and non-transformed cells lysates. The purified *C. rugosa* lipase enzyme did not show hydrolytic activity on pNPA substrate (Figure 2B). These results showed that, pNPA, a substrate used for different esterase enzymes (21), is hydrolyzed by intracellular esterases other than YpmR and lipase enzymes.

Effect of NaCl on Enzymatic Activity of *B. licheniformis* YpmR

In this study, the enzyme activity of a hypothetical YpmR enzyme belonging to the moderately halophilic *B. licheniformis* was investigated. Therefore, the recombinant YpmR enzyme synthesized in *E. coli* cells, is believed to be active in relatively high salt concentrations. Based on this assumption, the hydrolysis activity of the enzyme expressed in *E. coli* JM107 cells was tested at salt concentrations of 0.1 M, 0.5 M and 2.5 M. The maximum enzyme activity was obtained at 0.5 M NaCl concentration. It was observed that the enzyme lost approximately 50% activity at 2.5 M NaCl concentration. The YpmR enzyme was found to be more tolerant against increasing salt concentration as compared to the *C. rugosa* lipase enzyme used as reference (Figure 3).

Effect of pH on Enzymatic Activity of *B. licheniformis* YpmR

The activity of *B. licheniformis* YpmR enzyme synthesized in *E. coli* JM107 strain on pNPL substrate in Tris-$CaCl_2$ buffer at different pH was determined through spectrophotometry (Figure 4). The enzyme was found to have a low level of activity at pH 6 but to show a higher activity between pH 7-9.5. The maximum activity of the enzyme was obtained at pH 8. In comparison to the *C. rugosa* lipase enzyme, the *B. licheniformis* YpmR enzyme was found to be much more active at pH 9.5.

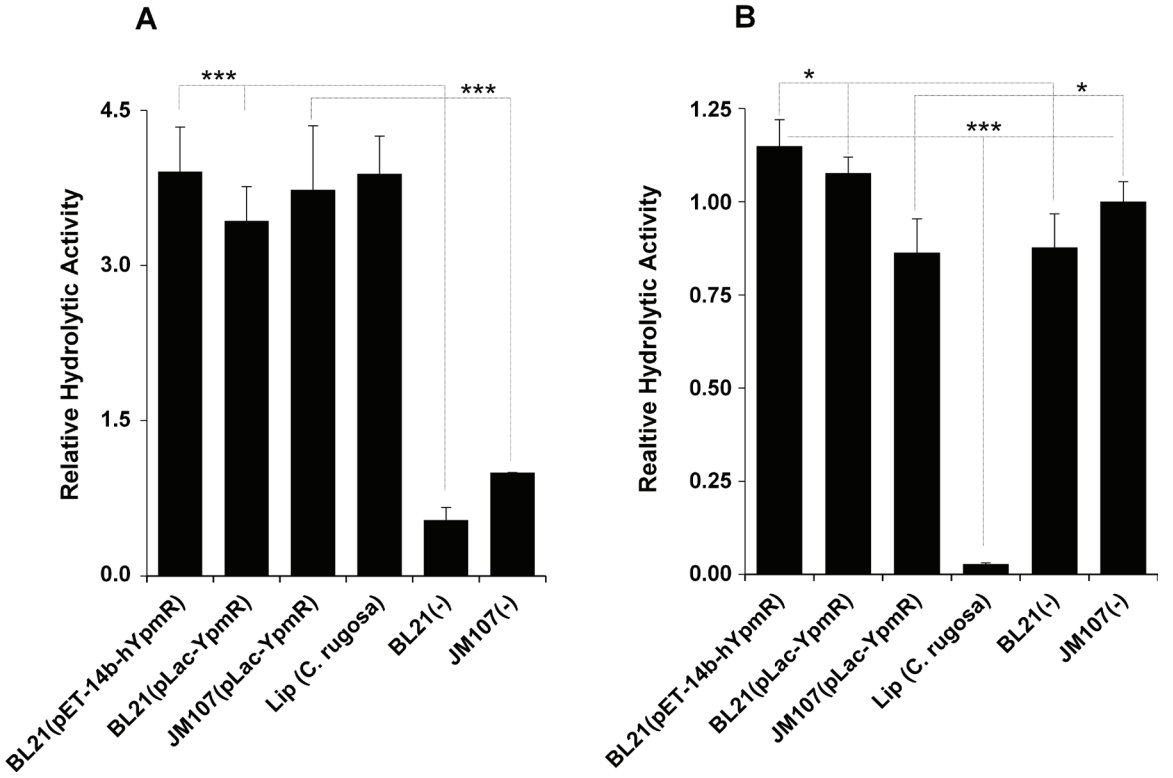

Figure 2. Hydrolytic activities shown by *B. licheniformis* hypothetical YpmR enzyme heterologously expressed in *E. coli* BL21 and JM107 bacterial strains in Tris-$CaCl_2$ buffer (pH 7), at 37°C, using pNPL and pNPA substrates. In the experiments, 15 ng (30 µl) of *C. rugosa* lipase enzyme (~700 U/mg) was used as a reference. Results are presented in terms of mean±SD (n = 5); (*) - P≥0.05; (***) - P≤0.01.

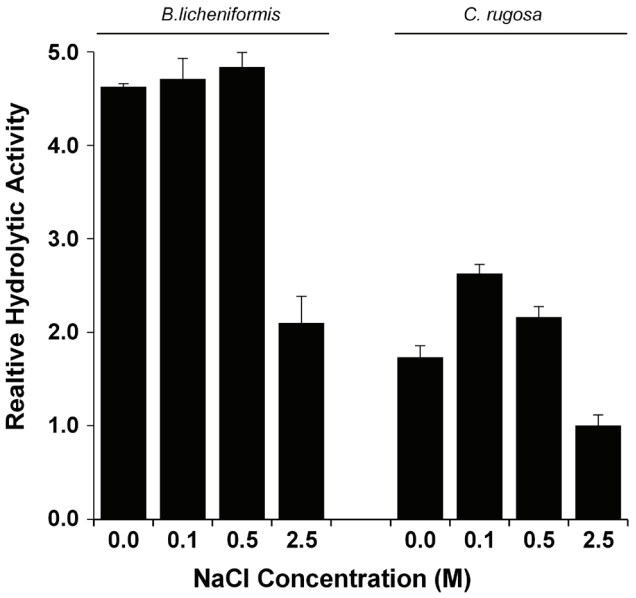

Figure 3. Hydrolytic activities of the hypothetical YpmR enzyme of *B. licheniformis* synthesized in *E. coli* JM107 strain and *C. rugosa* lipase enzyme on pNPL substrate at different salt (NaCl) concentrations. The experiments were performed at 37°C in Tris-$CaCl_2$ (pH.7) buffer containing different concentrations of NaCl. Results are presented in terms of mean±SD (n = 3).

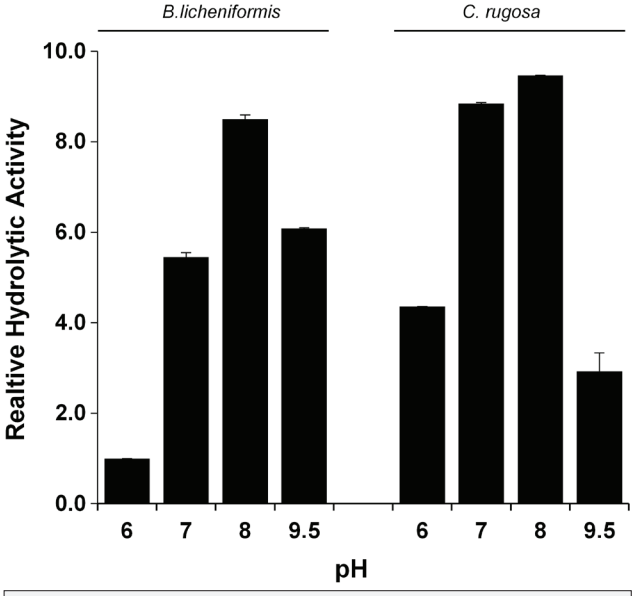

Figure 4. Hydrolytic activities of *B. licheniformis* YpmR enzyme synthesized in *E. coli* JM107 strain and *C. rugosa* lipase enzyme on pNPL substrate at different pH values. The experiments were performed at 37°C in Tris-$CaCl_2$ buffer at different pH. Results are presented in terms of mean±SD (n = 3).

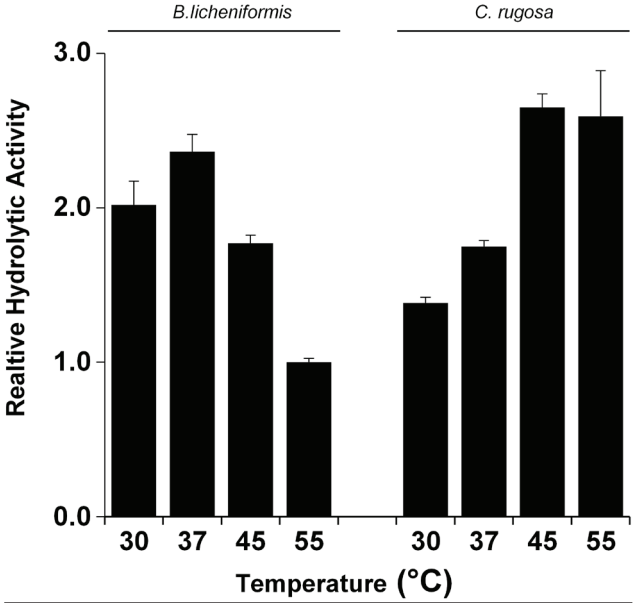

Figure 5. Hydrolytic activities of YpmR enzyme synthesized in *E. coli* JM107 strain and *C. rugosa* lipase at different temperature on pNPL substrate. The experiments were carried out using the cell lysate and 15 ng (30 µl) of *C. rugosa* lipase enzyme at different temperatures as per the values on the figure in Tris-$CaCl_2$ buffer at pH 7. Results are presented in terms of mean±SD (n = 3).

Enzymatic Activity of *B. licheniformis* YpmR at Different Temperatures

The activity of the YpmR enzyme was tested at 30°C, 37°C, 45°C and 55°C temperatures. The catalytic activity of the enzyme produced in *E. coli* JM107 cells on pNPL substrate at specified temperatures was determined using spectrophotometry (Figure 5). The enzyme showed high enzymatic activities at 30°C, 37°C and 45°C but the activity decreased by approximately 50% at 55°C. However, it was observed that the *C. rugosa* lipase enzyme used as reference showed maximum hydrolytic activity at 45-50°C.

Enzymatic Activity of *B. licheniformis* YpmR in the Presence of Triton X-100

One of the industrial applications of hydrolytic enzymes is the detergent industry. In this regard, it is of particular importance that lipase enzymes are active in the presence of detergent. Therefore, the activity of the *B. licheniformis* YpmR enzyme produced in *E. coli* JM107 was tested in the presence of Triton X-100, a non-ionic detergent, at varied concentrations. The pNPL hydrolysis activity of the hypothetical YpmR enzyme in 0.01-1% concentrations of Triton X-100 was determined using spectrophotometry. The enzyme maintains its activity up to 0.25% Triton X-100 concentration. At a concentration of 0.5% Triton X-100, the enzyme still shows approximately 50% enzymatic activity and the activity decreases to 25% in 1% Triton X-100 concentration (Figure 6). *B. licheniformis* YpmR enzyme was found to have higher tolerance against Triton-X-100 as compared to *C. rugosa* lipase enzyme.

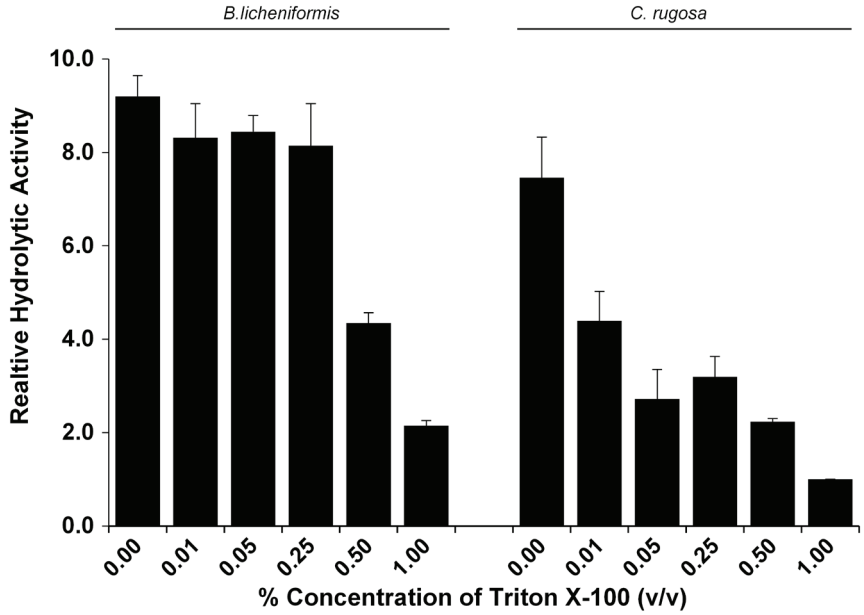

Figure 6. Hydrolytic activities of YpmR enzyme synthesized in *E. coli* JM107 strain and *C. rugosa* lipase at different temperature on pNPL substrate. The experiments were carried out using the cell lysate and 15 ng (30 µl) of *C. rugosa* lipase enzyme at different temperatures as per the values on the figure in Tris-$CaCl_2$ buffer at pH 7. Results are presented in terms of mean±SD (n = 3).

DISCUSSION

Hydrolytic enzymes such as esterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) are very important enzymes for biotechnological applications. Lipases preferentially hydrolyse insoluble triglycerides while esterases hydrolyse water-soluble esters (22). These enzymes are frequently used as biocatalysts in industrial fields such as the food industry, the detergent industry, and alternative energy production. As a result, hydrolytic enzymes that are active in extreme conditions are of great importance. In this study, the hydrolytic activity of YpmR enzyme of a moderately halophilic bacteria *B. licheniformis* was investigated. Based on *B. licheniformis* ATCC 14580 genome analysis, the hypothetically defined YpmR protein was identified as member of the SGNH hydrolase superfamily (https://www.ncbi.nlm.nih.gov/protein/52003893). However, there is no concrete data regarding its hydrolytic activity. Therefore, in order to determine the hydrolytic activity of *B. licheniformis* YpmR hypothetic enzyme, the gene was cloned in bacterial expression vectors and subsequently synthesized as a recombinant protein into *E. coli* cells. The synthesis of YpmR protein in the transformed cells was demonstrated by SDS-PAGE analyses (Figure 1). The hydrolytic activities of cell lysates containing the YpmR protein were tested using pNPL and pNPA substrates. Interestingly, from the experiments to determine the esterase group enzymatic activities using the pNPA substrate (23-26), high levels of hydrolytic activities were detected in both the plasmid transformed bacterial cell lysates and the untransformed cell lysate used as a negative control. As a standard control used in this study, *Candida rugosa* lipase enzyme showed no enzymatic activity against pNPA substrate. Therefore, the high activity detected in the transformed cell lysates is most likely due to endogenous esterases. The detection of extracellular esterase activity in *B. licheniformis* has also been reported in other studies (27-29). In contrast, the enzymatic activity experiments using pNPL as the substrate have detected hydrolytic activity in the YpmR expressing cell lysates, while very low levels of activity were measured in non-transformed cell lysates (Figure 2). While the *C. rugosa* lipase has no effect on pNPA substrate, it has shown to have hydrolytic effect on pNPL substrate. Therefore, like other true lipase enzymes, the experimental results obtained in this study show that the hypothetical *B. licheniformis* YpmR enzyme has hydrolytic activity on pNPL substrate indicating high preference for medium and long chain triglycerols substrates. This result corroborates with other studies (30-33).

The ability of enzymes to preserve their structural properties and enzymatic activities in different environmental conditions particularly increases their industrial importance (34). The stability of the YpmR enzyme synthesized in *E. coli* cells was tested at different pH values, salt (NaCl) concentrations, temperatures and in the presence of a non-ionic detergent Triton X-100. Based on the test results, the pH range in which YpmR enzyme showed the highest activity was at 7.0 - 9.5, while a 70-80% reduction in the enzyme activity was recorded at pH 6. In comparison to the *C. rugosa* lipase enzyme, it was concluded that the maximum activity of the synthesized YpmR enzyme was slightly more basic (Figure 4). In this respect, the *B. licheniformis* YpmR enzyme can be seen as a little more advantageous for industrial applications. The pH range in which the hypothetical *B. licheniformis* YpmR enzyme remains stable as reported in this study is similar to the pH values reported elsewhere (30,35-37).

One of the most important factors that affects the activities of enzymes is environmental temperatures. In this regard, proteins and enzymes from thermophilic bacteria living in hot springs have much more stable structures and, as a result, have high economic values (38-41). However, enzymes from halophilic bacteria are not expected to withstand very high temperatures. By evaluating the enzymatic activities of the YpmR enzyme containing cell lysates on pNPL substrate at a temperature ranging from 30°C to 55°C, it was revealed that the maximum activity of this enzyme was obtained at a temperature range of 30-45°C with an average of 50% activity reduction at 55°C. The maximum activity was observed for *C. rugosa* lipase used as a control at a temperature range of 45-55°C (Figure 5). This result showed that moderate halophilic *B. licheniformis* YpmR enzyme has a potential application in the detergent industry where the maximum temperature for most reactions is below 60°C. The impact of temperature on the enzyme activity of the recombinant *B. licheniformis* YpmR reported in our study corroborates with that of Selvin *et al.* 2012 and Annamalai *et al.* 2011 (30,36).

The maintenance of enzymatic activity at high salt concentrations by enzymes is of great importance in industrial applications. For this reason, many studies have been conducted on lipase enzymes from diverse halophilic organisms (42,43). Lipase enzymes isolated from many halophilic bacteria have exhibited the ability to withstand high salt concentrations (44). The *B. licheniformis* YpmR enzyme synthesized in *E. coli* cells have shown maximum activity using pNPL substrate at 0.5 M NaCl concentration. When compared to *C. rugosa* lipase enzyme, the YpmR enzyme was found to be more tolerant to high salt concentrations (Figure 3). Similar results of salt tolerant lipase enzymes have been recorded elsewhere (30,31,35).

The enzymatic activity of the YpmR enzyme on pNPL substrate was also tested in the presence of a non-ionic detergent, Triton X-100. The test results showed that the *B. licheniformis* YpmR enzyme retains its enzymatic activity in media containing about 0.01 to 0.25% Triton X-100. However, even in very low concentrations such as 0.05% Triton X-100 medium, the enzymatic activity of *C. rugosa* lipase enzyme, on average decreased by 60-70% (Figure 6). These data have highlighted the potential applicability of *B. licheniformis* YpmR enzyme as an additive in detergents. Reports about the stability of other *Bacillus* lipases in the presence of nonionic surfactants like Triton X-100 have been documented by other studies (31,33,35,45).

CONCLUSION

The moderate halophilic *B. licheniformis* hypothetical YpmR enzyme heterologously synthesized in *E. coli* cells has hydrolytic activity on pNPL substrate. The enzyme was observed to be more tolerant to an increase in NaCl and Triton X-100 concentrations compared to the *C. rugosa* lipase enzyme used as a control and showed potential for application in the detergent industry. Therefore, further purification and characterization of this promising hypothetical enzyme are important for future research goals.

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