ORIGINAL ARTICLE



Enzymatic utilization of oil and lignocellulosic biomass using halophilic marine bacteria *Micrococcus luteus* and *Pseudoalteromonas peptidolytica*

Jervian Johnson¹ · Kwon-Young Choi^{1,2}

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Abstract

In this study, hydrolytic and oxidative activities of enzymes isolated from halophilic microbes were characterized and applied for biomass utilization. First, lipase from *Micrococcus luteus*, and peroxidase and laccase from *Pseudoalteromonas phenolica* and *Pseudoalteromonas peptidolytica* were selected and their catalytic activities were determined, respectively. The *M. luteus* lipase encoding gene was synthesized after codon-optimization and could be successfully expressed in *Escherichia coli* with the assist of the Tif chaperone protein. The purified enzyme showed 119.13 ± 7.18 and 34.42 ± 5.91 U/mL of lipase and esterase activities, respectively. Moreover, the *M. luteus* lipase was applied for hydrolysis of the triglycerides mixture, which resulted in 182.9 ± 11.1 mg/L/h of glycerol productivity. Next, peroxidase and laccase activities of *P. phenolica* and *P. peptidolytica* were determined, and extracellular enzymes of *P. peptidolytica* was applied for lignocellulosic biomass degradation, which resulted in $91.9 \mu g$ glucose/mg lignocellulose of production yields. Finally, the hydrolytic and oxidative activities of the enzymes from halophilic microbes could be further utilized for biomass treatment and biochemical production.

Keywords Esterase · Peroxidase · Halophilic organism · High salt resistance · Lignocellulosic biomass

Introduction

Recently, due to concerns with respect to global warming, research on the production of various useful biochemicals from biomass sources has gained attention. Moreover, petrochemical-based chemical production leads to environmental pollution and human hazards which is also a major concern. Therefore, the need for the production of high-value-added chemicals from various sources of biomass has helped develop efficient bioprocesses, which can overcome the price competitiveness present in the chemical industry (Ma et al. 2012; Monlau et al. 2014; van Kuijk et al. 2015).

Research on efficient biomass treatment using ecofriendly bioprocesses has also gained importance (Sudheer

Kwon-Young Choi kychoi@ajou.ac.kr

¹ Department of Environmental Engineering, College of Engineering, Ajou University, Suwon, Gyeonggi-do, South Korea

² Department of Environmental and Safety Engineering, College of Engineering, Ajou University, Suwon, Gyeonggi-do, South Korea et al. 2017, 2018). One of the most promising carbon sources present in biomass is sugar-based carbohydrates (Crawford and Crawford 1976; Xu et al. 2019; Park and Choi 2020; Park et al. 2020). For example, for a long time, glucose has been commonly used and is the most important carbon source for microbial bioprocesses (Monlau et al. 2014). Although glucose can be obtained inexpensively from sugar cane or corn, recently, several studies have attempted to obtain glucose from lignocellulose (Elmore et al. 2020; Wang et al. 2018). Due to the complex structure of lignocellulose, enzymes such as peroxidases or laccases are required to convert it into glucose through oxidative degradation (Xu et al. 2019; Kong et al. 2017; Iiyoshi et al. 2017; Ladeira Azar et al. 2018). To date, several peroxidases and laccases have been identified and strategies for their production at commercial and industrial levels have been developed. In addition to carbon utilization, several lignin monomers have been utilized for the production of biopolymers, sensors, and electric materials and dyeing of textiles (Ahn et al. 2019; Araújo et al. 2018; Dayi et al. 2018).

Besides glucose, glycerol is another high-priority carbon source that can be used in microbial bioprocesses (Sudheer et al. 2018). Glycerol can be obtained either by chemical



synthesis from petroleum-based resources or biological processes such as microbial fermentation or biocatalytic conversion of vegetable oils. In such a biological process, hydrolytic biocatalysts such as lipases have played a key role in the generation of glycerol from various oil bioresources. Not only glycerol could be used for microbial carbon nutrients, but also be utilized for the preparation of several biochemicals such as C3-derivatives of 1,3-propanediol, 1,2-propanediol, lactic acid, and acrolein (Numpilai et al. 2021; Jiang et al. 2021; Zhang et al. 2018). Therefore, microbial and biocatalyst-based glycerol production has attracted great attention in biomass to platform biochemical research.

Previously, we isolated and characterized several halophilic species that could produce hydrolytic enzymes to hydrolyse carbohydrates, proteins, and bio-oils from a variety of bioresources in the presence of high salt conditions (Johnson et al. 2017). The halophilic strains included Marinobacterium, Marinobacter, Psudoalteromonas, Halomonas species which were all isolated from various coast and seawater environments (Johnson et al. 2017). Among the examined halophilic strains, Micrococcus luteus MLu-01 showed the highest extracellular lipase activity, while two Pseudoalteromonas species namely, Pseudoalteromonas phenolica PPH-01 and Pseudoalteromonas peptidolytica PPE-01 were reported to have high peroxidase and laccase activity (Table 1). In particular, the extracellular lipase activity of M. luteus, protease activity of P. phenolica, and amylase activity of P. peptidolytica were stably maintained at a concentration of 2 M NaCl (Johnson et al. 2017, 2018).

However, heterologous production and application of the halophilic hydrolase enzymes were not attempted yet. Therefore, in this study, lipase enzymes from *M. luteus* were first produced in *Escherichia coli* heterologous host system and its catalytic activity was determined. As the attempt to express each gene in *E. coli* resulted in a low expression level or insoluble fraction, chaperone protein of Tif in the pTf16 vector was additionally co-expressed to obtain a soluble fraction of target proteins. Next, peroxidase and laccase activities of *P. phenolica* and *P. peptidolytica* were also investigated. Finally, the solubilized lipase enzyme and extracellular laccase enzyme were examined for their ability to hydrolyze triglycerides mixture and oxidize lignocellulosic biomass to obtain glycerol and glucose, respectively. The results obtained from this study could be further utilized to treat a variety of biomass through bioprocessing to obtain economical carbon feedstocks such as food, pulp, and agricultural wastes.

Materials and methods

Chemicals and microbial species

Chemicals used for the lipase and esterase assays were purchased from Sigma-Aldrich (Sigma-Aldrich, South Korea). The lipase assay kit (MAK046), serum triglyceride determination kit (TR0100), peroxidase assay kit (MAK092), laccase assay instructions, laccase (L2157), *p*-nitrophenyl octanoate ester (21,742), triglyceride reagent (T2449), free glycerol reagent (F6428), syringaldazine (S7896), and lignocellulose (471,003) were also obtained from Sigma-Aldrich (the number in parentheses is product number) (Johnson et al. 2017, 2018). The microbial species, *Micrococcus luteus* MLu-01, *P. phenolica* PPH-01, and *P. peptidolytica* PPE-01, were used for lipase, peroxidase, and laccase activity assays, were obtained from Korean Collection for Type Cultures, Daejeon, South Korea (Johnson et al. 2017, 2018).

Cell growth medium and culture conditions

The growth media used for *M. luteus, P. phenolica*, and *P. peptidolytica* culture was marine broth (MB) as was described previously (Johnson et al. 2017, 2018). The seed culture was prepared in 2 mL of MB and incubated at 37 °C. For the main culture, the seed culture of each halophilic species was inoculated into 50 mL of MB (1% v/v) and incubated at 37 °C to obtain desired biomass.

Heterologous expression of *M. luteus* lipase and SDS-PAGE analysis

The encoding gene of *M. luteus* lipase (ML lipase) was amplified using primers, which were further cloned into the pET-22b(+) expression vector. The codon-optimized ML lipase gene was synthesized and cloned into the same vector (Bioneer, Daejeon, South Korea). Each pET-22b(+)::lipase

Table 1 Isolation and information of halophilic strains from environments

Species	Isolation information	Characteristics	Ref
Micrococcus luteus MLu-01	High salt culture of Graciliba- cillus bigeumensis	Lipase activity	Johnson et al. (2017)
Pseudoalteromonas phenolica PPH-01	Seawater	Protease activity	Johnson et al. (2017, 2018)
Pseudoalteromonas peptidolytica PPE-01	Seawater of Yamato Island, Sea of Japan	Amylase and chitinase activity	Johnson et al. (2017)



plasmid was transformed into *E. coli* BL21(DE3) for expression. Information about strains and genes were listed in Table 2.

The seed cultures were prepared by inoculating cells in 2 mL LB broth with ampicillin (100 μ g/mL), followed by incubation for 12 h at 37 °C. The cells were then sub-cultured by inoculating the seed culture into 50 mL LB broth (2% v/v), and the cells were incubated at 37 °C until an absorbance of 0.8 at 600 nm was obtained (Johnson et al. 2018; Sudheer et al. 2017). To induce ML lipase expression, four different IPTG concentrations (0.05, 0.1, 0.5, and 1.0 mM) were examined. After induction, the cells were further incubated at 30 °C for 12 h. The cells were then collected by centrifugation $(8000 \times g)$ for 15 min and washed twice with 5 mL of tris-buffered saline solution (Tris-Cl, 100 mM; NaCl, 150 mM; pH 8.8). The cells were then lysed using ultra-sonication. The total and soluble fractions of ML lipase were separated by centrifugation at $12,000 \times g$ for 10 min. The total and soluble fractions were prepared for SDS-PAGE analysis.

Co-expression of ML lipase with Tif chaperone and purification of ML lipase

ML lipase was co-expressed with a chaperone, as it enabled proper protein folding. The expression system using *E. coli*

BL21(DE3) was constructed by co-transformation of pET-22b(+)::*lipase* and a commercial chaperone plasmid pTf16 (Takara Bio) (Johnson et al. 2018; Sudheer et al. 2017). The expression of Tif was induced by adding 2 mg/mL L-arabinose solution. The resulting culture was then incubated at 37 °C until an absorbance of 0.8 was obtained at OD 600 nm. ML lipase was induced by the addition of IPTG as described previously. The culture was further incubated at 30 °C for an additional 12 h to enable co-expression of ML lipase was purified using a Ni–NTA purification kit (QIA-GEN Korea Ltd, Korea). The purified ML lipase enzyme was further studied for its catalytic activity.

Purified ML lipase and esterase activity assays

Analyses were performed using a lipase activity assay kit (CAS no. MAK046, Sigma-Aldrich), which uses a coupled enzyme reaction that results in a product that can be measured calorimetrically (570 nm) which is proportional to the enzymatic activity present in the sample (CAS no. MAK047, Sigma-Aldrich, South Korea) (Johnson et al. 2017). One unit of lipase was defined as the amount of enzyme that generates 1.0 μ mol of glycerol from triglycerides per minute at 37 °C (CAS no. MAK046, Sigma-Aldrich). For the esterase activity assay, short-chain *p*-nitrophenyl octanoate ester was used

 Table 2
 Information of strains, plasmids, DNA, and primers for DNA amplification

Strains	Relevant information	Reference/sources
E. coli strains		
DH5a	$F^{-}\phi$ 80lacZ M15 endA recA hsdR(rk ⁻ mk ⁻) supE thi gyrA relA Δ (lacZYA ⁻ argF) U169	
BL21(DE3)	B F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+] K-12(λS)	
Plasmids		
pET-22b (+)	pBR322 ori, Km ^R	Novagen
pET-22b (+):lipase	pET-28a (+) overexpressing lipase enzyme	This study
pTf16:: <i>tif</i>	pTf16 chaperone plasmid expressing Tif protein	Takara
Codon optimized lipase sequence	ATGCCACGGCCAGTTGCTGTAGCGCCAGAAGATTTGCCTCTTCGCTTAATGCCTGCGGCA CCGGCGAGACGTGACCATTGCGTAGTATTGTTACATGGGTTTACTAGTACCCCTGCCAGC GTGCGCGCATGGGCGGAAGGTCTTGCGGCCGGAGGATCACCGGTCTCAGTACCTCTG CTTCCCGGGCACGGGACTAGATGGGAAGATTTGGCCGCTACCGGAGCTGACGAGATA CGGGCCGCCGTGCGCGGGGGGGGGG	This study
Primers		
Forward	GGAGATATACATATGCCACGGCCAGTTGCTGAAA	This study
Backward	GTGGTGGTGGTGGTGGTGAGTCGTCATAGCGGCAAC	This study

as a substrate. Similarly, one unit of esterase was defined as the amount of enzyme that generates $1.0 \,\mu$ mole of nitrophenyl from *p*-nitrophenyl octanoate ester per minute at 37 °C (Johnson et al. 2017).

Peroxidase and laccase activities of *P. phenolica* and *P. peptidolytica* lysate

The P. phenolica and P. peptidolytica cells were incubated as described previously (Johnson et al. 2017). The culture supernatant and lysate obtained after sonication were used as extracellular and intracellular crude enzyme solutions, respectively. Peroxidase and laccase activity assays were carried out using the crude enzyme solutions. Analyses were performed using a peroxidase activity kit (CAS no. MAK092, Sigma-Aldrich). Peroxidase present in the sample catalyzes the reaction between H_2O_2 and the probe, thus resulting in a colorimetric (570 nm)/fluorometric ($\lambda_{ex} = 535$ / $\lambda_{em} = 587$ nm) product, proportional to the peroxidase activity present according to the manufacturer's protocol (CAS no. MAK092, Sigma-Aldrich). One unit of peroxidase is defined as the amount of enzyme that reduces 1.0 µmol of H₂O₂ per minute at 37 °C (CAS no. MAK092, Sigma-Aldrich). For the laccase activity assay, syringaldazine was used as a substrate and the assay was carried out according to the manufacturer's protocol (Sigma-Aldrich). One unit was defined as producing a 0.001 at OD₅₃₀ per minute at pH 6.5 at 30 °C in a 3 mL reaction volume using syringaldazine as the substrate (Sheikhi et al. 2012).

Lignocellulose degradation and glucose assay

The *P. phenolica* and *P. peptidolytica* cells were incubated for 12 h and 48 h, respectively. After incubation, varying concentrations of lignocellulose (0.05, 0.1, 0.5, and 1.0 wt%) were added to the growth medium. The concentration of the glucose released was monitored after 1 h and after 24 h of addition of lignocellulose, using the glucose assay sensor commercially available (Wang et al. 2018; Elmore et al. 2020).

Results and discussion

Hydrolytic enzymes isolated from halophilic species

In our previous report, we have screened a variety of halophilic species such as *M. luteus*, *P. phenolica*, *P. peptidolytica*, *Halomonas socia*, *Exiguobacterium aurantiacum*, and several *Marinobacter* species, which could produce high lipase, protease, amylase, cellulase, mannanase, chitinase, and xylanase activities (Johnson et al. 2017). Interestingly, the extracellular lipase (ML lipase) activity of *M. luteus*



MLu-0 isolated from a high-salt culture of *Gracilibacillus bigeumensis* was the highest, and intracellular amylase (PPE amylase) activity of marine bacterium *P. peptidolytica* PPE-01 was highest among all the halophilic strains examined in Table 1. Besides, *P. phenolica* PPH-01 isolated from seawater exhibited high extracellular protease activity, and its catalytic activity was well characterized using the purified enzyme (Johnson et al. 2018).

Particularly, the extracellular lipase activity of *M. luteus*, protease activity of *P. phenolica*, and amylase activity of *P. peptidolytica* were stably maintained at a concentration of 2 M NaCl. Apart from their hydrolytic activities, the crude extract of *P. phenolica* and *P. peptidolytica* also produce oxidative enzymes, such as peroxidase and laccase (Johnson et al. 2017). Therefore, it was worth constructing ML lipase producing heterologous strain and utilizing those halophilic hydrolases and oxidative enzymes in the treatment of vegetable oil and lignocellulose bioresources.

Expression and purification of lipase from *M. luteus* MLu-01

The ML lipase-encoding gene with 2,857 bp was amplified from *M. luteus* genome and cloned into the pET-22b(+) vector for expression. The induction condition was optimized by varying the concentration of 0.05 to 0.5 mM of IPTG. However, SDS-PAGE analysis revealed little or no expression of lipase (26.75 kDa) in both the total and soluble fractions (Fig. 1A). Therefore, the encoding gene was synthesized based on *E. coli* codon bias and attempted for ML lipase expression (Table 1). However, not much improvement in the expression was observed in spite of codon optimization and IPTG induction optimization (Fig. 1B).

The expression of the synthetic lipase gene was then attempted by co-expressing a chaperone protein as we were able to express halophilic protease enzyme isolated from *P. phenolica* successfully in the previous study after trying several solubilization attempts such as chemical and molecular chaperones (). Among the examined chaperones, Tif protein in the pTf16 vector, which is commercially available by Takara Chaperone Competent Cell BL21 series, could successfully express and solubilize the ML lipase (Fig. 2A). After obtaining the soluble fraction of the protein, the lipase was purified using a Ni–NTA His-tag purification kit (Fig. 2B). The purified enzyme was further studied by western blot analysis (Fig. 2C).

Determination of the catalytic activities of ML lipase and glycerol production from triglycerides

The catalytic activities of the purified ML lipase were investigated. The lipase and esterase activities were assayed by measuring glycerol and *p*-nitrophenol released from the





Fig. 1 Protein expression analysis by SDS-PAGE. A Wild-type ML lipase expression by varying the concentration of IPTG. B Synthetic ML lipase expression after codon optimization based on *E. coli* codon-biases

IPTG Concentration (mM)

hydrolysis of the triglycerides and *p*-nitrophenyl octanoate ester (Fig. 3A, B), respectively. The lipase and esterase activities were observed to be 119.13 ± 7.18 U/mL and 34.42 ± 5.91 U/mL, respectively. This is very promising compared to the values reported previously. For example, the lipase and esterase activities obtained in the commercial lipase (Sigma-Aldrich, CAS 9001-62-1) that was used as a positive control were found to be 278.23 and 62.75 U/ mL, respectively. In addition, the intracellular lipase activity of 12.85 mU/mg reported previously was in a range similar to that determined in this study (Johnson et al. 2017). Although the observed activities were approximately 50% of the commercial lipase, ML lipase could be utilized in various industrial applications, especially in high-salt environments in whole-cell systems.

Based on the calculated activity of ML lipase, the rate of glycerol produced from the degradation of fat was found to be 182.9 ± 11.1 mg/L/h at 37 °C. These results demonstrate the promising application of the ML lipase enzyme for the utilization of oil biomass to produce fatty acids and glycerol.

Determination of peroxidase and laccase activities of *P. phenolica* PPH-01 and *P. peptidolytica* PPE-01

In addition to ML lipase characterization, the extracellular peroxidase and laccase activities of the two halophilic strains *P. phenolica* PPH-01 (PPH) and *P. peptidolytica* PPE-01 (PPE) were also investigated. First, extracellular peroxidase and laccase activities were determined. The extracellular peroxidase activities of PPH and PPE were determined to be 13.2 and 40.7 U/g, respectively. However, the intracellular peroxidase activities were found to be 62.3 and 140.3 U/g, respectively, which were much higher than the extracellular activity. Interestingly, the intracellular peroxidase activity of PPE increased significantly up to 163.6 U/g (2.62 times) when the PPE cell culture was spiked with lignocellulose, which was a potential target for oxidative degradation (Fig. 4A).

Their extracellular laccase activities were determined next. The extracellular laccase activity in PPH and PPE was found to be higher, i.e., 2000–3000 U/mg, compared to the slight intracellular activity observed (Fig. 4B).

Oxidation of lignocellulose by fermentation with *P. peptidolytica* PPE-01

The PPE whole cells, which showed a higher peroxidase and laccase activity than PPH, were used for lignocellulose degradation. As their peroxidase activities were significantly induced by the supplementation of lignocellulose biomass, the crude extract of PPE cells was investigated for lignocellulose degradation activity. The degradation activity was calculated by monitoring the amount of glucose released during bioconversion of lignocellulosic biomass. The PPE cells were incubated for 18 h and 42 h, and the degradation reactions were initiated after the addition of 0.05, 0.1, 0.5, and 1.0 wt% lignocellulose. After 1 h and 24 h of incubation post-addition of lignocellulose, glucose concentrations were measured (Fig. 5). The initial cell culture incubation time did not significantly affect lignocellulose degradation; however, it was found that a longer reaction time could lead higher amount of glucose release. Also, the released glucose yields varied depending on the initial lignocellulose concentration, and the average yield was calculated to be approximately 91.9 µg glucose/mg lignocellulose used.



Fig. 2 A Construction of ML lipase and Tif chaperone coexpression system in *Escherichia coli*. **B** Soluble expression of synthetic ML lipase with the assistance of molecular chaperone Tif in pTf16 vector. **C** Western blot analysis of the ML lipase



According to previous results which produced glucose from lignocellulosic biomass, the glucose yields varied depending on bioconversion processes and biocatalysts used.



It is worthy to note that the additional supply of glucosidase enzyme, lignin pretreatment, and xylose recovery greatly affected glucose yields and recovery. As a crude extract of **Fig. 3** Lipase activity scheme. For quantitative analysis of lipase and esterase activity, glycerol **A** and *p*-nitrophenol **B** released from triglycerides and *p*-nitrophenyl octanoate ester, respectively, were monitored during enzyme reaction



PPE cells was used, hydrolase enzymes such as cellulase and xylanase enzymes, which extracellular activities were reported as less than 2 U/g, might be also slightly involved in the lignocellulose degradation (Johnson et al. 2017). To obtain desirable glucose yield, another engineering strategies to facilitate degradation and hydrolysis of lignocellulosic biomass will be necessary.

Conclusion

In this study, hydrolytic enzymes isolated from halophilic strains were produced and applied for biomass utilization. Firstly, the ML lipase enzyme was attempted to purify from halophilic *M. luteus*. However, it was not well expressed in *E. coli* and the codon-optimized lipase-encoding gene was synthesized. Furthermore, the synthetic gene was co-expressed with the chaperone protein of Tif, which resulted

in the soluble expression of ML lipase by western blotting assay. The catalytic activities of purified ML lipase as lipase and esterase were determined as 119.13 ± 7.18 and 34.42 ± 5.91 U/mL using triglycerides mixture and *p*-nitrophenyl octanoate substrates, respectively, which showed high lipase and esterase activities, which could have further applications for glycerol production from oils.

Next, two enzymes of peroxidase and laccase produced by halophilic strains, i.e., PPH and PPE, were investigated for their intra- and extracellular activities. Moreover, the PPE peroxidase enzyme was applied for lignocellulose degradation and showed approximately 91.9 μ g glucose/mg lignocellulose of glucose production. The halophilic species could hydrolyze bio-oils and biodegrade lignocellulose biomass, which is a promising renewable energy source. Moreover, it was also resistant to high salt environments. Thus, the halophilic species could be useful for high salt biomass processing and various industrial applications in the future.



Fig. 4 A Intracellular and extracellular activities of peroxidase enzymes produced by halophilic PPH and PPE strains. Catalytic activities of enzymes produced by PPH and PPE strains induced by lignocellulose supplementation were also determined. **B** Extracellular laccase activities of PPH and PPE strains







Fig. 5 Lignocellulose degradation activities of halophilic PPE cells at different lignocellulose concentrations, incubation time, and reaction time. The glucose released during incubation was monitored



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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval and consent to participate This article does not contain any studies involving human or animal participants.

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