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Research Article

Isolation and identification of lignin-degrading bacteria with laccase activity from the gut of the leopard moth, *Zeuzera pyrina* (Lepidoptera: Cossidae)

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Abstract: Gut bacterial symbionts have an essential role in the nutrition and fitness of xylophagous insects. These bacteria produce several enzymes like cellulase and laccase which are important in industrial applications. In this study, laccase-producing bacteria were isolated and identified from the gut of the wood borer leopard moth. Four novel laccase positive strains were isolated using guaiacol-containing agar plates. Among the strains of dc4f, le2f, lc2, and lb8, the strain le2f displayed high laccase activity of 0.059 U ml^{-1} toward syringaldazine as a typical laccase substrate. The isolates were identified based on biochemical tests and 16S rRNA gene sequencing analyses. Nucleotide BLAST analyses of 16S rRNA gene sequence exhibited that the strains of dc4f, lb8, lc2, and le2f, had the most similarity (with more than 98% identity) with *Enterobacter* sp. strain W-6 16S (ACCN: MK505390), *Serratia liquefaciens* strain N112 (ACCN: MK629784), *Brevibacterium* sp. strain 773 (ACCN: MH777897) and *Staphylococcus sciuri* strain KSI 708 (ACCN: KC113150), respectively. Overall, the current study is the first research on alkaliphilic bacterial strains from the gut of leopard moth with laccase activity.

Keywords: Biological degradation, Lignocellulose, Insect symbionts, Delignification

Introduction

Insects with more than one million species on Earth are the most diverse branch of the animal kingdom that exist in almost all habitat conditions with various food nutrition availability (Prasad *et al*., 2018). For many insects, lignocellulose is the main food source and they digest woody material using cellulolytic and ligninolytic enzymes that are produced by their gut-associated bacteria (Sun and Scharf, 2010).

Lignocellulose is the most abundant and sustainable biomass all over the world with a global availability for the production of biofuels (Clarke and Trinnaman, 2004). The framework of lignocellulose is composed of three organic compounds, including cellulose, hemicellulose, and lignin (Chen, 2014). Bioethanol is produced from the carbohydrate component of lignocellulose by diverse microbial communities in fermentation processes (Jönsson *et al*., 2013). In biofuel production as a first step, lignin is separated from the carbohydrate component and then sugars from enzymatic hydrolysis of cellulose and hemicellulose are converted into ethanol via fermentation (Dien *et al*., 2003).

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Lignin is a non-repeating polymer with a high-molecular-weight composed of three aromatic compounds: trans-coniferyl, trans-sinapyl alcohol and trans-p-coumaryl which is highly recalcitrant and resistant against enzymatic hydrolysis (Hergert, 1971). Microorganisms such as fungi and bacteria have a critical role in the bio delignification of lignocellulose. Although fungi produce efficient enzymes for lignin degradation, these enzymes are not stable under harsh conditions of pH and temperature and are not suitable for industrial applications (Martínez *et al*., 2005). Therefore, lignin-degrading bacteria with a wide range of pH and temperature stability have been isolated and frequently studied recently (Antonopoulos *et al*., 2001).

The leopard moth, *Zeuzera pyrina*, L. (Lep.: Cossidae) is the serious wood borer of many walnut orchards in Iran (Salari *et al*., 2015). The first instar larvae initially attack the young shoots and gradually bore into the tip of branches, while the larvae are developing, they penetrate the trunk and create feeding tunnels causing tree weakness and death in serious situations (Ashtari *et al*., 2011). The leopard moth has an alkaline gut an adaptation of a tannin-rich diet that creates harsh living condition for symbiotic bacteria (Prasad *et al*., 2018). So, the bacteria that survive in these conditions are useful for industrial applications. Many of the industrial processes, including bleaching and pulping are carried out under alkaline pH and require enzymes with high alkaline stability (Nimchua *et al*., 2012). Furthermore, in the last decade, insects as new sources of industrial bacteria have been considered by researchers (Krishnan *et al*., 2014).

Laccases (EC 1.10.3.2) are multi-copper oxidoreductase enzymes that are involved in aromatic compounds oxidation and delignification process (Couto and Herrera, 2006). Laccase has a high potential for industrial applications and can be used in many industrial scopes, including textile, food and pulp and paper industries (Couto and Herrera, 2006). The aim of this study was to isolate and identify alkalotolerant bacteria with laccase activity from the gut of the leopard moth. For this purpose, screening for laccase producing bacteria were performed on guaiacol-containing plates. Laccase activity assay was performed using syringaldazine, as a laccase substrate, and finally, the isolated bacteria were identified based on biochemical tests and 16S rRNA gene sequencing analyses

Materials and Methods

Gut isolation from the leopard moth larvae

Forty third instar larvae of leopard moth were collected from infected walnut branches in Saman County of the Chaharmahal and Bakhtiari Province in the west of Iran. Larval gut dissection was performed in completely sterile conditions under a stereomicroscope. The body of larvae was surface sterilized using 70% ethanol for one minute and then washed three times with sterile distilled water to remove residual alcohol. Each gut was transferred to a 1.5 ml microtube containing 200 µl of phosphate-buffered saline (PBS), buffer pH 7.

Isolation of lignin-degrading bacteria

Dissected digestive tract of the leopard moths were homogenized and spread on nutrient agar plates and were incubated at 27 °C and 37 °C for one week (Rezaei *et al*., 2017). To screen lignin-degrading bacteria single colonies were cultured on a modified solid basal medium (SBM) containing (gl^{-1}): K_2HPO_4, 7; KH_2PO_4, 3; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.1, kraft lignin (Sigma-Aldrich), 0.5 and agar, 15 (Cartwright and Holdom, 1973). Isolated strains in this step were screened for laccase producing bacteria.

Screening for Laccase-producing bacteria

Luria-Bertani (LB) agar medium, supplemented with 0.1% (v/v) of guaiacol was used to screen laccase producing isolates. Bacteria were cultured on guaiacol containing plates and were incubated at 30 °C for two weeks. Afterwards, formation of reddish brown zone around

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bacterial colonies was evaluated as laccase positive strains (Rezaei *et al*., 2017).

Biochemical analyses for identification of bacteria

Isolated laccase positive bacteria were primarily identified based on biochemical tests including gram staining (Murray *et al*., 1994), oxidase, and catalase reaction (Wood and Krieg, 1989), maltose, glucose and lactose fermentation, Tween 80 hydrolysis and hydrogen sulfide (H_2S) production (Garrity, 2012).

16S rRNA gene amplification for identification of bacteria

Selected strains' genomic DNA was extracted by a DNA extraction kit according to the manufacturer's instructions (CinnaGen, Teheran). Partial 16S rRNA genes were amplified from genomic DNA using the universal primers forward primer

16sF: 5′ AGAGTTTGATCCTGGCTCAG 3′ and reverse primer

16sR: 5′ACGGCTACCTTGTTACGACT 3′ (TAG Copenhagen, Denmark),as described by Weisburg *et al*. (Weisburg *et al*., 1991) in a Bio-Rad Mycycler Thermal cycler (Hercules, USA). The total volume of 25 µl PCR reaction mixture consisted of 12.5 µl of PCR mastermix, 1 µl of bacterial template DNA, 1 µl of forward and reverse primers (10 ρmol concentration) and 9.5 µl of deionized water. PCR amplification cycling was done as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 2 min and a final extension at 72 °C for 10 min. The quality of PCR product was evaluated using 1% agarose gel. 16S rRNA gene sequencing was carried out at the Bioneer Biotechnology Company (Seoul, South Korea) using the primers mentioned above. The obtained DNA sequence was compared with available gene data at National Center for Biotechnology Information (NCBI). Phylogenetic analysis was conducted utilizing the EzTaxon server and aligned with mega software version 6 (Kim *et al*., 2012).

Laccase activity assay

For assessing laccase production, the strains were cultured in nutrient broth medium (Merck) at 30 °C for 30 hours and then the culture medium was centrifuged at 12,000 × g for 20 min at 4 °C and the supernatant was used as laccase enzyme source. The activity of laccase was determined by measuring the absorbance of oxidized syringaldazine (SGZ) (Sigma-Aldrich), as substrate, at 520 nm. The reaction mixture was composed of 240 µl of 20 mM Tris-HCl buffer (pH 8), 30 µl of 1 mM SGZ, and 30 µl of bacterial culture supernatant. One unit of the laccase activity was defined as the amount of enzyme that can oxidize one µmol of the SGZ per minute (Siroosi *et al*., 2016).

Results

Leopard moth gut

The digestive tract of the leopard moth generally has a cylindrical structure consisting of three parts, foregut, midgut, and hindgut (Fig. 1). Midgut is separated from the foregut by gastric cecum in the anterior midgut. These ceca create a more surface area of the midgut to facilitate and upgrade the digestion and absorption of food. The hindgut starts from where the malpighian tubules are attached to the pyloric valve and extends to the anus.

Isolation and primary characterization of laccase producing bacteria

Only four strains showed laccase activity on solid medium containing guaiacol as a laccase indicator. These isolates were named as dc4f, lb8, lc2, and le2f. The reddish brown zone around bacterial colony indicates a positive activity of laccase (Fig. 2).

The results of biochemical tests for the initial identification of the isolates are shown in Table 1. Based on the results, all four strains were catalase positive, dc4f, and lb8 were gram-negative, and lc2 and le2f were gram-positive strains.

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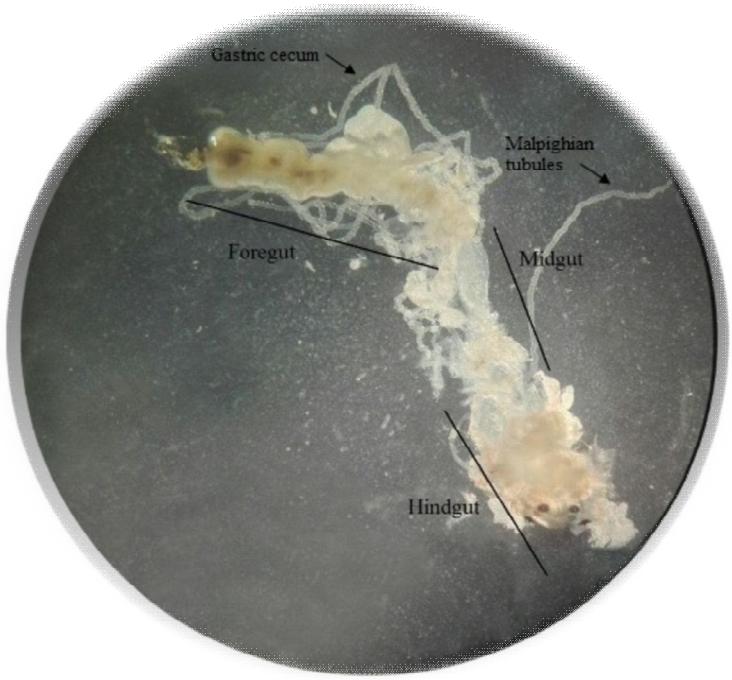

Figure 1 Digestive tract of *Zeuzera pyrina* (dorsal view) with three parts of the foregut, midgut, and hindgut.

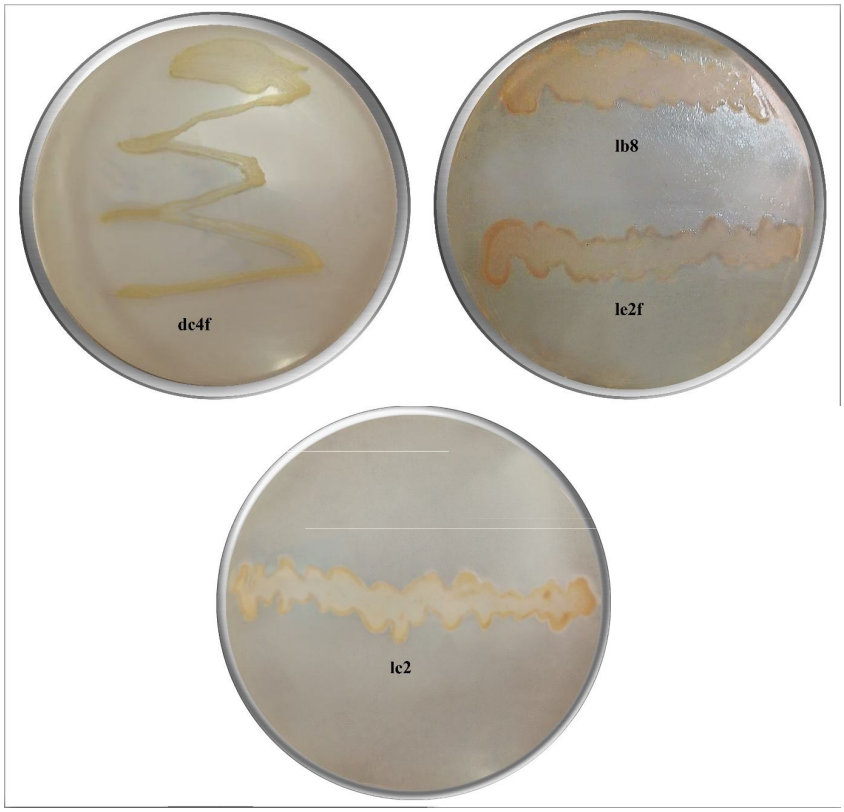

Figure 2 Bacterial colony of strains dc4f, lb8, lc2, and le2f showing reddish- brown halo around colony on 0.1% (v/v) guaiacol containing plates.

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Table 1 The biochemical characteristics of the laccase producing strains from the gut of the leopard moth.

Strain	Gram Staining	Catalase activity	Oxidase activity	Fermentation of glucose	Fermentation of maltose	Fermentation of lactose	H_2S production	tween 80 hydrolysis
le2f	+	+	+	+	-	-	+	-
lc2	+	+	+	+	-	-	+	-
lb8	-	+	-	+	+	-	+	+
dc4f	-	+	-	+	+	+	+	-

(+) refers to positive result and (-) refers to negative result.

Phylogenetic analysis and identification of the strains

The PCR product quality of 16S rRNA gene is shown in Fig. 3.Based on 16S rRNA sequence and phylogenetic analyses, all four strains had more than 98.0% sequence similarity to their nearest phylogenetic neighbor (Fig. 4). Strains of dc4f, lb8, lc2, and le2f were identified as *Enterobacter* sp., *Serratia* sp., *Brevibacterium* sp. and *Staphylococcus* sp., respectively according to their sequence comparison with previously recorded sequences and were deposited in GenBank database under the following accession numbers MN559365, MN559367, MN559368, and MN559369 respectively.

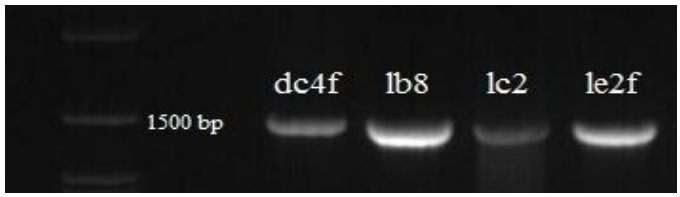

Figure 3 PCR products quality of isolates dc4f, lb8, lc2, and le2f on 1% agarose gel.

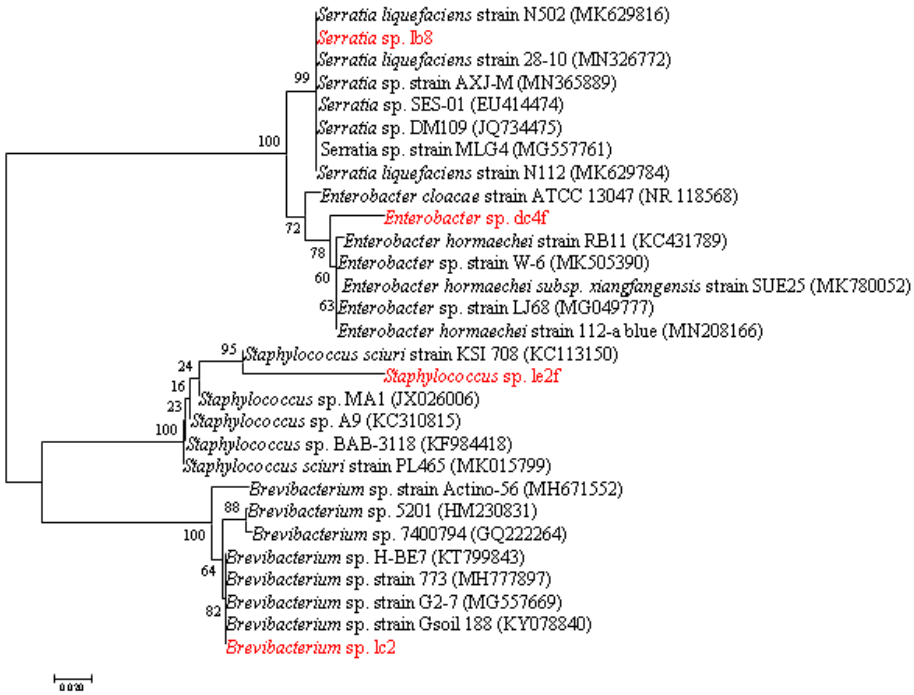

Figure 4 Phylogenetic analysis using neighbor joining method based on 16S rRNA sequences of bacterial isolates associated with digestive tract of the leopard moth. Strains from this study are highlighted in red.

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Laccase activity

Enzyme activity on SGZ as a substrate was measured spectrophotometrically by monitoring the increase of absorbance at 520 nm. The strain of le2f, with 0.059 U ml^{-1} activity, showed the highest enzyme activity compared to other strains. The other strains: lb8, dc4f, and lc2 showed 0.041, 0.039, and 0.036 U ml^{-1} activity, respectively.

Discussion

Many insect species benefit from associations with facultative and obligate nutritional symbionts to upgrade their nutrient imbalanced diet (Henry *et al*., 2015). This symbiosis allows insects to inhabit in many ecological niches that are inappropriate for other animals and feed on many undesirable foods to increase their population. The diet is the most crucial factor affecting the diversity and abundance of the gut microbial flora in insect species (Engel and Moran, 2013). In comparison to other insects, detritivores and xylophagous insects have high abundance of microbial flora in their gut due to complexity of their diet (Engel and Moran, 2013). Although various groups of microorganisms are present in the xylophagous insects' gut and contribute to digesting imbalanced food, these are the bacterial population that have had a critical role in woody material digestion and host physiological fitness (Prasad *et al*., 2018). It seems that the extreme alkalinity in caterpillars' gut caused harsh conditions to microbial colonization. However, many bacteria have survived under these conditions as alkaline tolerant bacteria (Engel and Moran, 2013). In this study, four laccase producing bacteria namely *Enterobacter* sp. dc4f (Proteobacteria, Enterobacteriaceae), *Serratia* sp. lb8 (Proteobacteria, Enterobacteriaceae), *Brevibacterium* sp. lc2 (Actinobacteria, Brevibacteriaceae) and *Staphylococcus* sp.le2f (Firmicutes, Staphylococcaceae) were isolated from the alkaline gut of the leopard moth. Previous studies showed that most of the screened bacteria from the caterpillars' gut belong to the Bacillaceae, Enterobacteriaceae, Staphylococcaceae, Enterococcaceae, and Pseudomonadaceae families. *Enterobacter*, *Staphylococcus*, and *Serratia* are three of the most common bacterial genera in the gut of lepidopteran species (Paniagua Voirol *et al*., 2018). *Enterobacter* sp. dc4f and *Serratia* sp. lb8 with lignin-degrading capacity in the leopard moth gut belong to γ-Proteobacteria and Enterobacteriaceae family. It is also demonstrated that members of the Enterobacteriaceae family are capable of degrading many constituents of lignocellulose, such as hemicellulose, cellulose, and lignin. Furthermore, *Enterobacter* and *Serratia* species have been frequently reported in wood-feeding insects (Hu *et al*., 2014). Grbic-Galic (1986) indicated that under anaerobic condition *Enterobacter* species were able to perform many reactions on ferulic acid (an aromatic lignin derivative) including dehydroxylation, O-demethylation, decarboxylation on aromatic rings and reduce the double bond in the side-chain. In addition, Zhou *et al* (2017) also isolated two lignin-degrading bacteria, *Enterobacter hormaechei* PY12 and *Bacillus licheniformis* MX5 from the gut of termite *Reticulitermes chinensis* Snyder which have high lignin peroxidase activity. All these results indicate that bacteria belonging to the phylum Proteobacteria play a crucial role in wood material degradation in the xylophagous insects. *Staphylococcus* sp. le2fis is the other laccase producing strain in the leopard moth gut. This strain with 0.059 U mL^{-1} activity showed the highest laccase activity in this study. *Staphylococcus* has also been reported as the major symbiotic bacterium genus in the gut of higher termite in Australia (Eutick *et al*., 1978). The fourth bacterial isolate of the leopard moth gut with significant laccase activity was *Brevibacterium* sp. lc2 which belongs to the Actinobacteria. In agreement with this report, the *Brevibacterium* sp. isolated from the adult gut of citrus long-horned beetle *Anoplophora chinensis* also had the capacity of both aromatic compound degradation and cellulolytic activity (Rizzi *et al*., 2013).

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In conclusion, in this study, gut-bacterial communities of the leopard moth were studied for the first time, and four strains with laccase activity were isolated and identified. These strains can be introduced as alkaline tolerant laccase producing bacteria that make them appropriate candidates for biotechnological processes and industrial applications.

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جداسازی و شناسایی باکتری‌های تجزیه‌کننده لیگنین با فعالیت لاکازی از روده کرم خراط *Zeuzera pyrina* (Lepidoptera: Cossidae)

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چکیده: باکتری‌های هم‌زیست با حشرات چوب‌خوار نقش مهمی در تغذیه و تناسب میزبانشان دارند. این باکتری‌ها بسیاری از آنزیم‌های مهم با کاربرد صنعتی از قبیل سلولاز و لاکاز را تولید می‌کنند. در این مطالعه باکتری‌های تولیدکننده آنزیم لاکاز از لوله گوارش کرم خراط جداسازی و شناسایی شدند. با استفاده از محیط کشت حاوی گوآیاکول چهار استرین جدید باکتریایی با فعالیت لاکازی جداسازی شدند. در بین چهار استرین dc4f، le2f، lc2 و lb8، استرین le2f با فعالیت لاکازی در حدود ۰/۰۵۹U/ml بر روی سوبسترای سیرینگالدازین بیش‌ترین فعالیت را از خود نشان داد. ایزوله‌ها با استفاده از تست‌های بیوشیمیایی و نیز توالی‌یابی ژن 16S rRNA شناسایی شدند. نتایج حاصل از بلاست نوکلوئوتیدی ژن 16S rRNA نشان داد که استرین‌های dc4f، lb8، lc2 و le2f به‌ترتیب دارای بیش‌ترین شباهت (بیش از ۹۸ ٪ شباهت) با گونه‌های *Enterobacter* sp. strain W-6 16S (ACCN: MK505390)، *Serratia liquefaciens* strain N112 (ACCN: MK629784)، *Brevibacterium* sp. strain 773 (ACCN: MH777897) و *Staphylococcus sciuri* strain KSI 708 (ACCN: KC113150) بودند. به‌طور کلی در این مطالعه برای اولین‌بار باکتری‌های قلیادوست با فعالیت لاکازی موجود در لوله گوارش کرم خراط مورد مطالعه قرار گرفته است.

واژگان کلیدی: تجزیه زیستی، لیگنوسلولز، هم‌زیست‌های حشرات، دلیگنیفیکاسیون

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