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Exploration of Novel Extracellular Xylanase-Producing Lactic Acid Bacteria from Plant Sources

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Abstract

Xylanases play a crucial role in bio-transforming sustainable agricultural polymers into xylose-based oligosaccharides, which have great potential in various biotechnology applications. Nevertheless, the application of bacterial xylanase is hindered by the high cost of developing recombinant bacteria to overcome the low activity and narrow pH stability. Considerable efforts have been made to discover and explore new wild bacterial strains that produce highly effective and environmentally sustainable extracellular xylanase enzymes for various targeted biotechnological and industrial applications. Lactic acid bacteria (LAB) have recently been proven to be versatile producers of extracellular hydrolytic enzymes. Therefore, this study aimed to isolate and characterise extracellular xylanase-producing LAB (EXLAB) from plant sources. The specific extracellular xylanase activity was determined across a wide pH range, from acidic to alkaline. Subsequently, the expression of xylanase genes of EXLAB grown under acidic and alkaline conditions was determined by quantitative reverse transcription polymerase chain reaction. A total of 45 putative LAB were isolated from radish, gundelia and rhubarb plants. They were identified by phenotypic and genotypic approaches. However, only 15 LAB isolates were confirmed as EXLAB. *Weissella confusa* and *Pediococcus pentosaceus* were the most common species among the identified EXLAB. The XylW (~196 bp) and XylP (189 bp) xylanase genes were then amplified from *W. confusa* and *P. pentosaceus*, respectively. *P. pentosaceus* G4 demonstrated the most versatile extracellular xylanase production that was active from pH 5 to pH 8. However, a significant increase in extracellular xylanase gene expression (13.45-fold) at pH 5 was noted as compared to pH 8. Similarly, *P. pentosaceus* G4 also exhibited the highest extracellular xylanase activity (0.88 U/mg) at pH 5. This study reveals the potential of *P. pentosaceus* G4 as an eco-friendly and novel extracellular xylanase producer possessing broad pH stability. The robust gene expression and activity of extracellular xylanase imply *P. pentosaceus* G4 is a promising candidate for sustainable enzymatic processes essential for the environmentally friendly enzymatic reactions and applications.

Keywords: lactic acid bacteria; extracellular xylanase; *Weissella confusa*; *Pediococcus pentosaceus*; qPCR; xylanase gene



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1. Introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria that appear in a coccus or rod shape. They are non-spore-forming and ferment carbohydrates to produce lactic acid as the predominant end-product [1]. LAB are generally considered non-pathogenic and non-toxic [2–4] and environmentally friendly microorganisms with specific enzymatic functions [5]. Hence, they have been deemed as Generally Regarded as Safe (GRAS) microorganisms. Furthermore, they possess a long track record for safe use across various industries, such as pharmaceuticals, food chemicals and as the biotransformation agent for lignocellulosic biomass [6–9]. LAB have garnered significant interest due to its vast potential to produce versatile extracellular hydrolytic enzymes, including xylanase [7,9–13]. Recently, Ali et al. [14] optimised the extracellular xylanase production of *Pediococcus pentosaceus* G4.

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1. x) responsible for catalysing the endohydrolysis of 1,4- β -D-xylosidic linkages of xylan (essential constituent of hemicelluloses) into xylose and xylooligosaccharides. The synergistic action of multiple xylanases would break down xylan effectively [15–17]. Xylanases are mainly divided into two functional groups. Endo-xylanases cleave the internal xylosidic links of the complex polysaccharide xylan [18]. Exo-xylanases hydrolyse long-chain xylo-oligomers from the reducing end to short-chain xylo-oligomers and xylose. The xylose yield is enhanced when both exo and endo-xylanases react together [19]. Xylanase enzymes are categorised based on substrate affinity, attributed to the amino acid sequences of their catalytic domains [15]. Xylanases are categorised into glycoside hydrolase (GH) families, namely GH 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, and 62, according to the Carbohydrate-Active Enzymes (CAZy) database (www.cazy.org). GH10 and GH11 are widespread xylanases that are determined by the hydrophobic clusters and the amino acid sequences in the catalytic module [16,20–22]. Environmental factors, including temperature and pH, significantly influence the activity and stability of xylanases. Most xylanases have a pH optimum between 4.0 and 9.0 and a temperature optimum of 30 to 60 °C, depending on the organism and the structural stability of the enzyme [23].

Xylanases are vital in several industrial applications [7]. They facilitate the conversion of xylan into xylose and xylooligosaccharides [16,17], providing potential advantages in diverse industries such as the food industry [24], animal feed industry [25], brewing industry, biofuel production, xylitol and ethanol production, saccharification of xylan to xylose, plant cell protoplast production, pigment and plant oil extraction, silage production, deinking waste paper, textile industries, biorefinery and the bleaching process of paper and pulp [20,23,26–29]. The capacity of xylanase to expedite several biochemical processes, enhance product quality and improve digestibility has led to a significant increase in its demand in recent years in sectors of animal feed and food industries [24,25,30]. However, the search for xylanase-producing bacterial strains capable of withstanding the high temperatures and across acidic and alkaline conditions remains a challenge [25].

Many organisms have been reported to produce xylanases, such as molluscs, snails, crustaceans, insects, rumen bacteria, cyanobacteria, protozoa, fungi and marine algae [31]. Recently, bacterial xylanases have gained high popularity, attributed to their faster growth rate, reduced space requirements, ease of maintenance and potential for genetic engineering of bacterial xylanases [32]. Bacteria have demonstrated an impressive ability to withstand challenging environmental conditions [33]. Hence, the ability of the bacteria to produce xylanases under such conditions has garnered significant interest [34].

Choosing non-pathogenic and nontoxic microbe strains is essential to ensure the safe production of xylanase by the producer strain, such as those that have been safely used in the food industry for a long history [35]. The safe and ecologically friendly

microorganisms that produce xylanases would warrant the safe application of xylanases across various sectors. Exploring xylanase-producing microbes, specifically LAB, offers a promising approach to addressing the safety and toxic challenges. Recent investigations have discovered that various LAB have the potential to produce extracellular xylanase, which broadens the microbial sources for xylanase enzyme producers [7]. Although LAB have been reported as promising candidates for xylanase production, attempts to identify environmentally friendly LAB that are capable of producing extracellular xylanase under broader pH conditions are very limited.

Understanding the gene expression profiles of xylanase-producing LAB is essential for comprehending their potential in industrial processes. Various molecular approaches can be employed to assess the expression of xylanase genes, including reverse transcription polymerase chain reaction (RT-PCR) [36]. Nevertheless, there is a dearth of extensive research on the expression of xylanase genes in LAB, underscoring the necessity for additional exploration in this field of study.

Hence, the objective of this study is to isolate extracellular xylanase-producing LAB (EXLAB) from plant sources and characterise the respective extracellular xylanase activity under various pH conditions. Additionally, the comparative expression of xylanase genes was assessed for the selected EXLAB grown in acidic and alkaline conditions. Understanding the extracellular xylanase production capacity and xylanase gene expression would facilitate the development of environmentally friendly EXLAB applications in various industrial settings.

2. Results and Discussion

2.1. Isolation, Identification, and Characterisation of the Extracellular Xylanase-Producing Lactic Acid Bacteria

A total of 45 putative LAB cultures were isolated from radish (*Raphanus sativus*), rhubarb (*Rheum rhabarbarum*) and gundelia (*Gundelia tournefortii*). Nevertheless, only 15 (33.33%) extracellular xylanase-producing lactic acid bacteria were detected using both qualitative and quantitative methods (Table 1). The plant source employed for the EXLAB isolation contains abundant xylan [18,37]. Hence, the selected plant sources could potentially harbour xylanase-producing bacteria.

The xylan composition of the lignocellulosic components of selected plant sources induces the production of xylanase enzymes [31,38]. Therefore, the environments within and surrounding the plants are likely to contain bacteria that have adapted and thrive to utilise xylan as an energy source, making them an ideal habitat for their growth. However, plants are diverse, and their cell walls are highly heterogeneous structures [39]. The nature of xylan differs across plant species [40]. Thus, selecting different types of plants may facilitate access to a broad array of microorganisms with potentially distinct xylanase-producing capabilities. Plants vary in their ability to support different species of xylanase-producing bacteria, each with distinct substrate specificities and biochemical characteristics. Therefore, isolating EXLAB from different sources will enhance the discovery of new xylanase-producing bacteria with improved features and capabilities for diverse industrial applications, including pulp and paper, feed production, detergents and textiles [41,42].

Our findings are consistent with previous reports of isolating xylanase-producing bacteria from plant sources. For instance, Suto et al. [43] isolated xylanase-producing bacteria from *Gnaphalium japonicum* and *Ulmus davidiana*. Seo et al. [44] found that xylanase was produced effectively by endophytic bacteria, *Bacillus* sp. isolated from the interior of radish leaves and roots. Furthermore, *Bacillus* sp. and *Glutamicibacter* sp. that were isolated from *Arthrocnemum macrostachyum* have the capability to produce industrially important xylanase [45]. These reports highlight the potential of plant-isolated bacteria as a

xylanase producer. Therefore, finding new plant sources to isolate EXLAB, particularly for industrial use, remains necessary.

Table 1. Lactic acid bacteria isolated from selected plant sources.

Plant Source	Total Lactic Acid Bacteria Number	Xylanase-Producing Lactic Acid Bacteria		
		Isolate Number	Isolate Label	% of Xylanase-Producing Bacteria
Radish	19	6	R1	31.57%
			R3	
			R4	
			R5	
			R6	
			R7	
Gundelia	12	5	G1	41.66%
			G4	
			G5	
			G6	
			G7	
Rhubarb	14	4	Rh8	28.57%
			Rh9	
			Rh11	
			Rh13	
Total	45	15		33.33%

2.1.1. Semi-Quantitative Determination of Extracellular Xylanase Activity of Lactic Acid Bacteria

A total of 45 putative LAB isolates were screened for their ability to produce extracellular xylanase enzyme using xylan agar medium. The LAB isolates were grown on xylan-containing MRS media. Subsequently, a Congo red stain was added to detect undegraded xylan, which formed a dark brown colour complex. Clear zones surrounding the bacterial suspension well imply the production of xylanase by the LAB isolates. The diameter of the clear zones was measured, and a total of 15 LAB isolates showed positive extracellular xylanase activity (Table 2) and were designated as the EXLAB.

Table 2. Semi-quantitative analysis of extracellular xylanase-producing lactic acid bacteria isolated from plant sources.

Sources	Isolate No.	Clear zone Diameter (mm)
Radish	R1	5.2 ^d ± 0.1154
	R3	4.1 ^f ± 0.0577
	R4	4.03 ^f ± 0.0333
	R5	5.33 ^d ± 0.1452
	R6	5.83 ^b ± 0.1452
	R7	3.03 ^g ± 0.0333
Gundelia	G1	4.7 ^e ± 0.0577
	G4	6.53 ^a ± 0.0881
	G5	5.2 ^d ± 0.1154
	G6	5.7 ^{bc} ± 0.0577
	G7	6.5 ^a ± 0.1154
Rhubarb	Rh 8	6.3 ^a ± 0.0577
	Rh 9	4.7 ^e ± 0.0577
	Rh 11	4.53 ^e ± 0.1452
	Rh 13	5.43 ^{cd} ± 0.0881

Values are mean ± SEM, *n* = 3. Means ± SEM within the same column that share a common superscript (a–g) are not significantly different (*p* > 0.05).

This findings demonstrated that the 15 EXLAB bacterial isolates could utilise beechwood xylan as a carbon source by producing extracellular xylanase enzymes. Bacterial isolates with a clear zone of hydrolysis have been classified as good extracellular xylanase producers (Figure 1). The EXLAB G4, isolated from gundelia, produced the largest clear-zone diameters (6.53 mm) among the studied EXLAB isolates. In comparison, the smallest clear zone diameter of xylan hydrolysis (3.03 mm) was noted for the EXLAB R7 isolate.

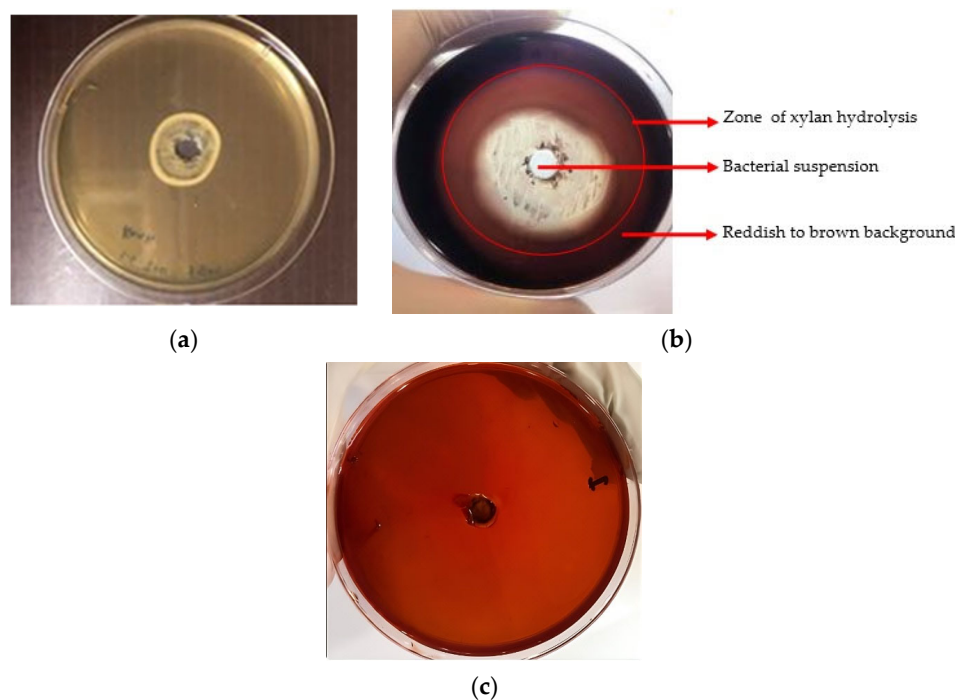


Figure 1. Representative semi-quantitative screening of extracellular xylanase activity of lactic acid bacteria isolated from plant sources using MRS medium supplemented with xylan. (a) growth of xylanase-producing bacteria on MRS medium containing xylan. (b) A clear zone of xylan hydrolysis surrounding the well, as observed with the growth of the G4 isolate, stained with Congo red dye. (c) A negative control of *Lactococcus lactis* showed no clear zone of xylan hydrolysis.

Our finding agrees with earlier studies. Shrestha et al. [46] used the same screening method to reveal the potential of *Streptomyces* sp. for xylanase enzyme production. Moreover, the largest zone of hydrolysis on the xylan agar plate has confirmed that *Bacillus tequilensis* UD-3 has the capability of producing xylanase enzyme [47]. In addition, the bacteria *Bacillus subtilis* S1C6 and *Bacillus pumilus* S2C5 have been demonstrated to form hydrolysis zones when streaked over xylan-containing media, as reported by Pandey and Gupta [48]. Moreover, the *Streptomyces* sp. isolates (SSA3, SSA7, SSA19, and SSA20) grown on xylan agar medium hydrolyse xylan, as evidenced by the clear zones around the colonies against a red background. The plate screening process also revealed that *Bacillus sonorensis* T6 possessed xylanolytic activity, as evidenced by the formation of a white halo surrounding the colonies [49]. Based on these findings, the plate assay technique is a practical preliminary method for semi-quantitative assessment of xylanase-producing bacteria.

2.1.2. Quantitative Determination of Extracellular Xylanase Activity of Lactic Acid Bacteria

In this study, a total of 15 EXLAB isolated from plant sources showed positive extracellular xylanase activity. The extracellular xylanase activity of 15 EXLAB isolates was determined quantitatively at pH 5, 6.5 and 8. The extracellular xylanase assay was performed by using the cell-free supernatant (CFS) of EXLAB isolates cultured in de Man,

Rogosa, Sharpe (MRS) broth medium. Figure 2 illustrates that the specific extracellular xylanase activities of the 15 EXLAB isolates varied substantially from acidic to alkaline pH conditions. Generally, the specific extracellular xylanase activities detected for all studied EXLAB were significantly ($p < 0.05$) higher at pH 5 as compared to pH 6.5 and 8, respectively. Although all studied EXLAB isolates produced substantial extracellular xylanase activity at pH 5 (Figure 2a), different specific extracellular xylanase activities were observed for different EXLAB isolates. In comparison, the highest specific extracellular xylanase activity (0.88 U/mg) was detected for the G4 EXLAB isolated from the gundelia plant at pH 5, whereas the R7 EXLAB isolated from the radish plant demonstrated the lowest specific extracellular xylanase activity of 0.36 U/mg.

Most of the studied EXLAB demonstrated extracellular xylanase activity at pH 6.5, except for the G7 EXLAB, which displayed no extracellular xylanase activity (Figure 2b). Interestingly, the G4 EXLAB also exhibited the highest specific xylanase activity of 0.47 U/mg at pH 6.5 (Figure 2b), as compared to the EXLAB R4 isolate, which demonstrated the lowest extracellular xylanase activity of 0.29 U/mg. At pH 8, the G5 EXLAB showed the highest specific extracellular xylanase activity of 0.29 U/mg, whilst the R7 EXLAB displayed the lowest extracellular xylanase activity of 0.16 U/mg (Figure 2c). It is noteworthy that both R5 EXLAB and Rh8 EXLAB displayed no extracellular xylanase activity at pH 8, as illustrated in Figure 2c.

The findings in this study implied that the EXLAB isolated from gundelia plants produced significantly greater specific extracellular xylanase activity as compared to EXLAB isolated from other plant sources. Furthermore, the EXLAB isolates generally demonstrated significantly higher ($p < 0.05$) specific extracellular xylanase activity in acidic conditions and the lowest specific extracellular xylanase activity was detected under alkaline conditions.

The significantly higher specific extracellular xylanase activity observed in EXLAB isolated from gundelia could be attributed to the distinctive lignocellulosic composition, particularly the hemicellulose content, compared to other plant sources. The recent study of Rozhgar [50] revealed a notable carbohydrate content of 12.67% in fresh samples of gundelia from the Mawat region (Sulaimani, Iraq). The considerably high carbohydrate concentration suggests a potentially greater lignocellulosic structure, such as hemicellulose, including xylan content, as indicated by the markedly high extracellular xylanase activity of EXLAB isolated from the gundelia plant. Radish exhibits a maturation-dependent alteration in polysaccharide composition, marked by a substantial increase in xylan composition [51]. Thus, it is a xylan-rich plant. Rhubarb has been shown to contain hemicellulose in its pomace; however, the exact amount of xylan remains undetermined [52]. Therefore, a detailed biochemical analysis of the hemicellulose composition in selected plant sources is essential to understand and correlate plant-microbe extracellular xylanase enzymatic activities and interactions.

LAB are microaerophilic bacteria known for their ability to produce lactic acid from various carbohydrates through a fermentation process, which occurs in anaerobic and facultative anaerobic conditions. Heterofermentative or homofermentative LAB have been reported [53–55]. Their application in the fermented food industry has gained significant traction [56], with an ongoing exploration of their potential as starter cultures in the food industry and as bio-preservative agents, which is attributed to their ability to extend the shelf life of fermented foods by creating acidic conditions through the biosynthesis of lactic acid [57,58] from the carbohydrate fermentation process.

Although xylanolytic fungi and bacteria are well-documented, the occurrence of extracellular xylanase enzymes produced from LAB isolated from plant sources has not been previously reported. In the present study, we investigated the capability of LAB isolated from hemicellulose-rich plant sources to produce versatile extracellular xylanase

enzymes that are active in a broad pH range, from acidic to alkaline pH conditions. Indeed, the findings of the current study demonstrated the production of extracellular xylanase enzyme by the studied EXLAB isolates over a broad pH range, spanning from acidic pH 5 to alkaline pH 8. Therefore, they may play a promising role in the degradation of the polysaccharide component of the renewable hemicellulose biomass. Our results also highlighted that the studied EXLAB could be considered a promising choice for producing extracellular xylanase enzymes. These enzymes offer numerous benefits, such as cost-effectiveness with the adoption of renewable agro biomass as substrates and safer production conditions, since LAB are generally regarded as safe (GRAS) microorganisms.

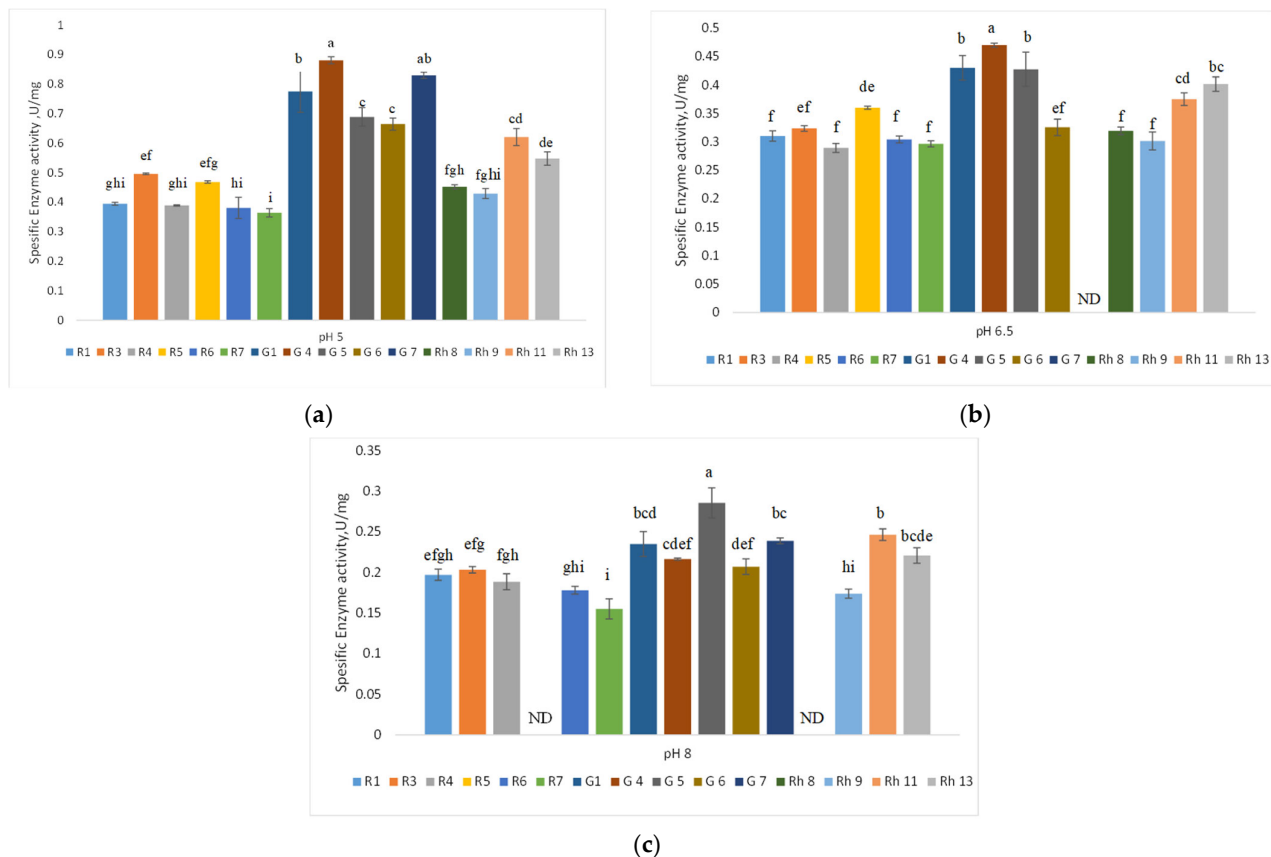


Figure 2. Effect of pH on specific extracellular xylanase activity produced by the presumptive LAB isolated from radish, rhubarb and gundelia samples. (a) Specific extracellular xylanase activity at pH 5. (b) Specific extracellular xylanase at pH 6.5. (c) Specific extracellular xylanase at pH 8. Bars represented with different lowercase letters are statistically significant at $p < 0.05$. ND = Not detected.

Typically, xylanolytic fungi are the leading industrial xylanase enzyme producers [59–62]. However, more bacterial species have been demonstrated to have the ability to produce xylanase, such as *Bacillus* species, which have been reported as notable xylanase producers [47,63–66]. As for LAB, Lee et al. [7] reported that the *Lactiplantibacillus plantarum* RS5 exhibited a maximum xylanase activity of 0.17 U/mg at pH 5. In contrast, the highest activities were observed at pH 6.5 and pH 8.0 for the *Lactiplantibacillus plantarum* RG14 (0.21 U/mg) and the *Lactiplantibacillus plantarum* B4 (0.15 U/mg). Moreover, Adiguzel et al. [10] found that *Pediococcus acidilactici* GC25 demonstrated its highest level of activity at a pH of 7. The versatile extracellular xylanase enzyme activities of the G4 EXLAB, which are active over a wide pH range, correspond with the finding of Zabidi et al. [9], who also reported that *Lactiplantibacillus plantarum* RI 11 exhibited hemicellulolytic enzyme activity across a broad pH range.

The results of the present study are consistent with previous studies that have investigated the effect of pH on xylanase activity. For instance, Fu et al. [67] reported that xylanase activity was stable at pH 5. Likewise, Olopoda et al. [61] demonstrated that the highest xylanase activity was noted between pH 4 and 5. In addition, Rajabi et al. [68] reported that maximum xylanase activity was detected at pH 5, but decreased as the pH increased. According to Malhotra and Chapadgaonkar [69], the xylanase activity of *Bacillus licheniformis* exhibited significant activity between pH 6.5 and 9.5. Furthermore, Ulucay, Gormez and Ozic [70] reported that the maximum xylanase activity of *Bacillus subtilis* occurred at pH 7. In contrast, Raj et al. [71] exhibited the highest xylanase activity of *Bacillus Licheniformis* at pH 9, while demonstrating a significant decline in activity at pH 4 and pH 5, respectively, implying that different xylanase producers exhibit different pH-dependent enzymatic activity.

Generally, the stability of enzymes at various pH conditions is determined by a spatially uneven arrangement of charged residues around the particular enzyme. The acid-stable xylanase exhibits a notable abundance of acidic residues on its surface, which is hypothesised to minimise the electrostatic repulsion between positively charged residues under low pH conditions, preserving its structural integrity and enzymatic activity. On the other hand, enzymes that exhibit stability in alkaline environments are commonly distinguished by reduced acidic residues and a higher amount of arginine [15]. In addition, a further distinction among various xylanase enzymes lies in their respective quantities of salt bridges. Salt bridges are electrostatic interactions formed between amino acids with opposite charges, which play a crucial role in maintaining the structural stability of enzymes. Acidophilic xylanases have a reduced number of salt bridges compared to alkaliphilic xylanases, primarily due to an increased number of negatively charged residues on their surface, which results in electrostatic repulsion [15].

This study demonstrated that the evaluated EXLAB isolates can produce xylanase enzymes, enabling the breakdown of xylan-containing materials across a wide range of pH conditions. The potential suitability of the EXLAB candidate for various industrial applications that specifically require both stability and activity within acidic environments, such as those used in the maceration and purification processes for producing fruit juices and wines. Additionally, the studied EXLAB also exhibited promising stability and activity in neutral or alkaline environments, making them potentially applicable to other relevant industrial processes. In comparison, the extracellular xylanase enzyme produced by G4 EXLAB holds considerable potential due to its enhanced enzymatic activity and stability at acidic pH conditions, as well as its exceptional tolerance over a broad pH range up to pH 8. These attributes make it a highly suitable candidate for many industrial applications [10]. Nevertheless, to gain a deeper understanding of the physicochemical characteristics and molecular structure of the extracellular xylanase enzyme, future studies should focus on the purified extracellular xylanases.

2.1.3. Phenotypic Characteristics of Extracellular Xylanase-Producing Lactic Acid Bacteria

Morphological and biochemical investigations showed that the EXLAB isolates R1, R3, R4 and R6 were Gram-positive coccobacilli (Figure 3). In contrast, EXLAB isolates R5, R7, G1, G4, G5, G6, G7, Rh8, Rh9 and Rh11 were observed as Gram-positive cocci under microscopic observation (Figure 4). The EXLAB colonies were white to creamy in appearance with circular forms and convex elevation. They were catalase-negative and produced lactic acid. They grew at a wide range of temperatures and tolerate salt concentrations up to 6.5% (*w/v*).

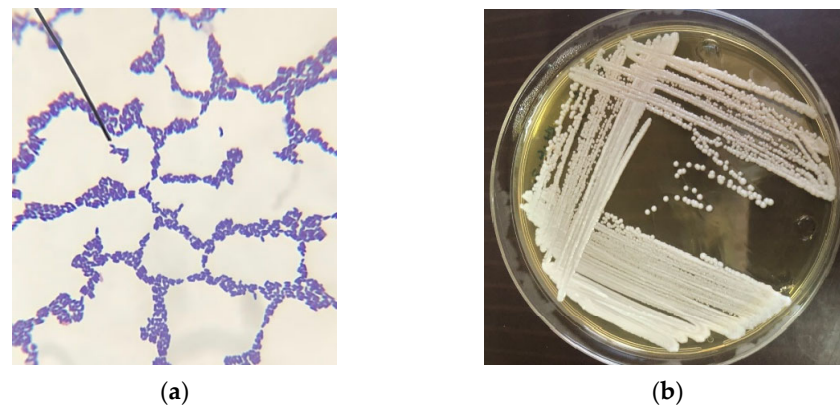


Figure 3. Representative micrographs of *Weissella confusa*. (a) Gram-positive coccobacilli; (b) Colony morphology on de Man, Rogosa and Sharpe agar.

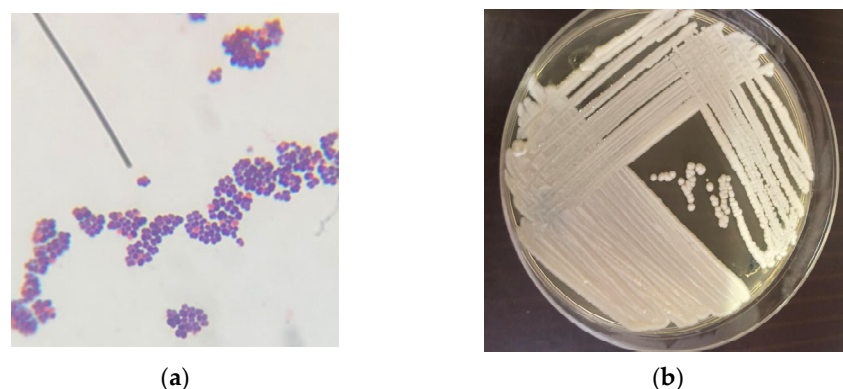


Figure 4. Representative micrographs of *Pediococcus pentosaceus*. (a) Gram-positive tetra cocci; (b) Colony morphology on de Man, Rogosa and Sharpe agar.

2.1.4. Genotypic Characterisation of Xylanase-Producing Lactic Acid Bacteria

The 16S rDNA gene is widely used for genotypic bacterial identification, providing species-level identification and enabling phylogenetic analysis [72]. Altogether, 45 putative LAB were isolated from radish, gundelia, and rhubarb plants. Nevertheless, only 15 putative LAB were confirmed as EXLAB after determining their specific extracellular xylanase activities, as shown in Figure 2. The 16S rDNA of EXLAB was amplified and sequenced for genotypic identification. A single band of 16S rDNA was detected on a 15% (*w/v*) agarose gel electrophoresis (Supplementary Figure S1).

Four EXLAB isolates (R1, R3, R4 and R6) were identified as *Weissella confusa* and 11 EXLAB isolates (R7, G1, G4, G5, G6, G7, Rh8, Rh9, Rh11 and Rh13) were identified as *Pediococcus pentosaceus*. The alignment of the 16S rDNA sequences with the NCBI database revealed that all the studied EXLAB exhibited sequence homology greater than 99%, for both *W. confusa* and *P. pentosaceus*. However, 16S rDNA sequences have limitations in discriminating closely related strains. Therefore, to differentiate EXLAB strains, whole-genome sequencing is required to identify strains of both species isolated in this study.

A phylogenetic tree was subsequently constructed using the Maximum Composite Likelihood (MCL) model in the MEGA programme by comparing the 16S rDNA sequences of EXLAB, as shown in Figure 5, which presents the Neighbour-joining phylogenetic tree used to determine the evolutionary relationship among the EXLAB strains of both *W. confusa* and *P. pentosaceus* obtained in this study. The genera and species observed in this study were found to be the predominant microorganisms that were capable of growing in MRS medium. Species of the genus *Pediococcus* exhibited rapid growth, often constituting the predominant proportion of LAB as compared to *Weissella* species.

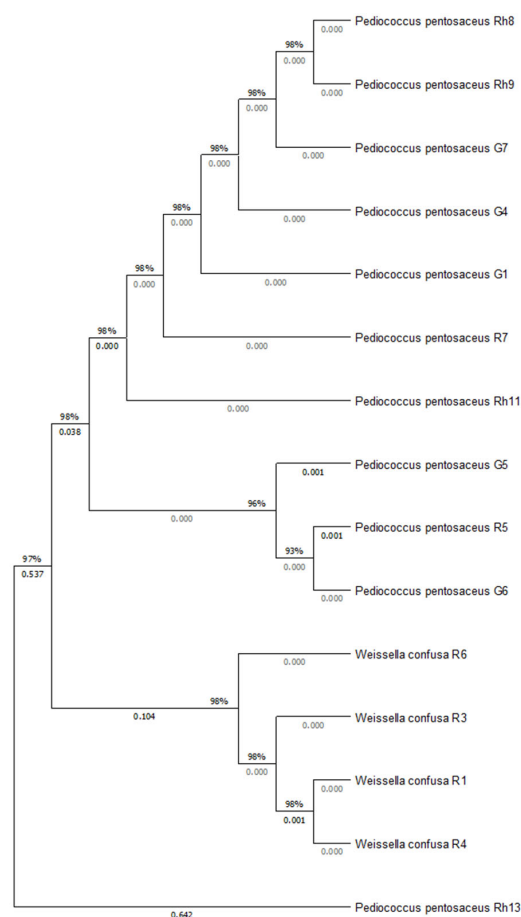


Figure 5. Neighbour-joining phylogenetic tree of *Weissella confusa* and *Pediococcus pentosaceus*.

2.2. Molecular Characterisation of the Xylanase Gene of Extracellular Xylanase-Producing Lactic Acid Bacteria

The 15 selected EXLAB were evaluated and confirmed for their xylanolytic enzyme production capability, demonstrating that they are xylanolytic bacteria via XylW and XylP gene expression analyses for both *W. confusa* and *P. pentosaceus* strains, respectively.

2.2.1. Detection of XylW and XylP Xylanase Genes

In this study, the XylW gene was employed to amplify the xylanase gene from the extracted genomic DNA of *W. confusa* strains with the following forward (F) primer: GGCTTCTTCAAGTGGTCAGC and reverse (R) primer: CGCCTTCTTCTTCATCCTTG. The results of the XylW gene detection were 100%, as all *W. confusa* strains were positive for the presence of the XylW xylanase gene. The amplified XylW gene, with a molecular weight of ~196 bp, was observed when analysed by 15% (*w/v*) agarose gel electrophoresis (Supplementary Figure S2).

As for the XylP gene detection, the F primer: TTTACCTGCCGTTACCCAAG and R primer: TGGGTGTTTTTGGTTTGACA were explicitly designed to amplify the XylP xylanase gene from the extracted genomic DNA of *P. pentosaceus* strains. The amplicon of the XylP amplification, with a predicted molecular weight of 189 bp, was successfully detected on 15% (*w/v*) agarose gel electrophoresis, where the band was positioned between the 100 bp and 200 bp bands of the 100 bp DNA ladder, suggesting the presence of this XylP xylanase gene, as shown in Supplementary Figure S2.

PCR amplification of both XylW and XylP xylanase genes produced amplicons of the expected sizes of 196 bp and 189 bp, respectively, confirming the presence of the respective xylanase genes in all studied strains. Identical amplicon sizes were observed

since both primers targeted the conserved region of the xylanase genes harboured in different xylanase-producing strains. Complete sequencing of the xylanase gene amplicons is vital for studying the diversity of xylanase genes.

Generally, the molecular characterisation of the LAB xylanase genes is limited. The presence of XylW and XylP xylanase genes among the 15 EXLAB confirmed that the studied *W. confusa* and *P. pentosaceus* strains were xylanolytic LAB. Hence, the EXLAB could be a potential resource(s) for xylanolytic enzymes. The results of the current study were similar to those of Lei et al. [13], who revealed *Pediococcus acidilactici* BCC-1 harboured xylan oligosaccharide (XOS) transportation genes, xylanase and xylosidase enzymes. In another study, Gilad et al. [73] reported that *Bifidobacterium animalis* subsp. *lactis* BB-12 encoded genes for xylanases and β -d-xylosidase enzymes when it grew on xylooligosaccharides. Interestingly, *Lactococcus lactis* 210, *Lactococcus lactis* IO-1 and *Lactococcus lactis* NRRL B-4449 have been demonstrated to be deficient in the xylan degradation pathway associated with the xylose operon [74]. However, genes encoding xylanolytic enzymes have been found in *Lactobacillus lactis* (*Lactobacillus delbrueckii lactis*) [75], indicating genetic diversities and strain-specific xylanase activity amongst the LAB.

2.2.2. Expression Analysis of XylW and XylP Xylanase Genes

The RT-qPCR method is the most accurate and sensitive for estimating gene expression. Therefore, it was established as the gold standard in gene expression studies. The concept of this technique relies on comparing the expression level of a particular gene among different treatments [76].

The present study investigates the impact of pH conditions, specifically pH 5 and pH 8, on the expression of XylW and XylP genes in EXLAB. The 15 EXLAB were cultured in MRS broth under pH 5 and pH 8 conditions, with pH 6.5 as a control. However, the normalisation of the expression of the targeted xylanase gene was conducted using housekeeping genes *recAW* and *recAP* for *W. confusa* and *P. pentosaceus*, respectively. The Ct values of the gene amplification were obtained from the RT-PCR programme, and the quantification was performed by calculating the Δ Ct value. Gene expression fold change was determined using relative quantification (RQ) based on the delta-delta Ct value.

As for *W. confusa* strains, the highest fold of XylW xylanase gene expression was observed for the *W. confusa* R3 strain (12.53) at pH 5. In contrast, the lowest XylW gene expression fold was demonstrated by *W. confusa* R6 strain (1.58), as shown in Figure 6a. Similarly, at pH 8, *W. confusa* R3 strain also showed the highest fold of XylW xylanase expression (0.85). In contrast, *W. confusa* R6 strain exhibited the lowest fold of XylW gene expression (0.07), as illustrated in Figure 6b. The xylanase gene expression results indicated that *W. confusa* strains grown at pH 5 showed significantly higher XylW gene expression fold than those grown at pH 8.

Similarly, this study also investigated the impact of acidic and alkaline conditions on XylP gene expression in *P. pentosaceus* strains. At pH 5, *P. pentosaceus* G4 strain demonstrated the highest fold of XylP gene expression (13.45), whereas *P. pentosaceus* R7 strain exhibited the lowest fold of XylP gene expression (0.16), as illustrated in Figure 6c. In contrast, at pH 8, the highest fold of XylP gene was observed for *P. pentosaceus* G5 strain (0.95), while *P. pentosaceus* R5 strain demonstrated the lowest fold of XylP gene expression (0.004), as shown in Figure 6d.

The findings of the XylW and XylP xylanase gene expression demonstrated a significant increase in the expression of either XylW or XylP genes by the 15 EXLAB strains under pH 5 growth conditions. Nevertheless, a reduction in the expression of the targeted xylanase genes was noted when the EXLAB strains were cultured in MRS broth at pH 8. Therefore, the acidic environment has significantly induced the expression of the studied

xylanase genes in all EXLAB strains, which is in line with the quantitative analysis of extracellular xylanase activity discussed in Section 2.1.2 and illustrated in Figure 2. These findings expand our understanding of how pH affects the expression of the xylanolytic gene in EXLAB.

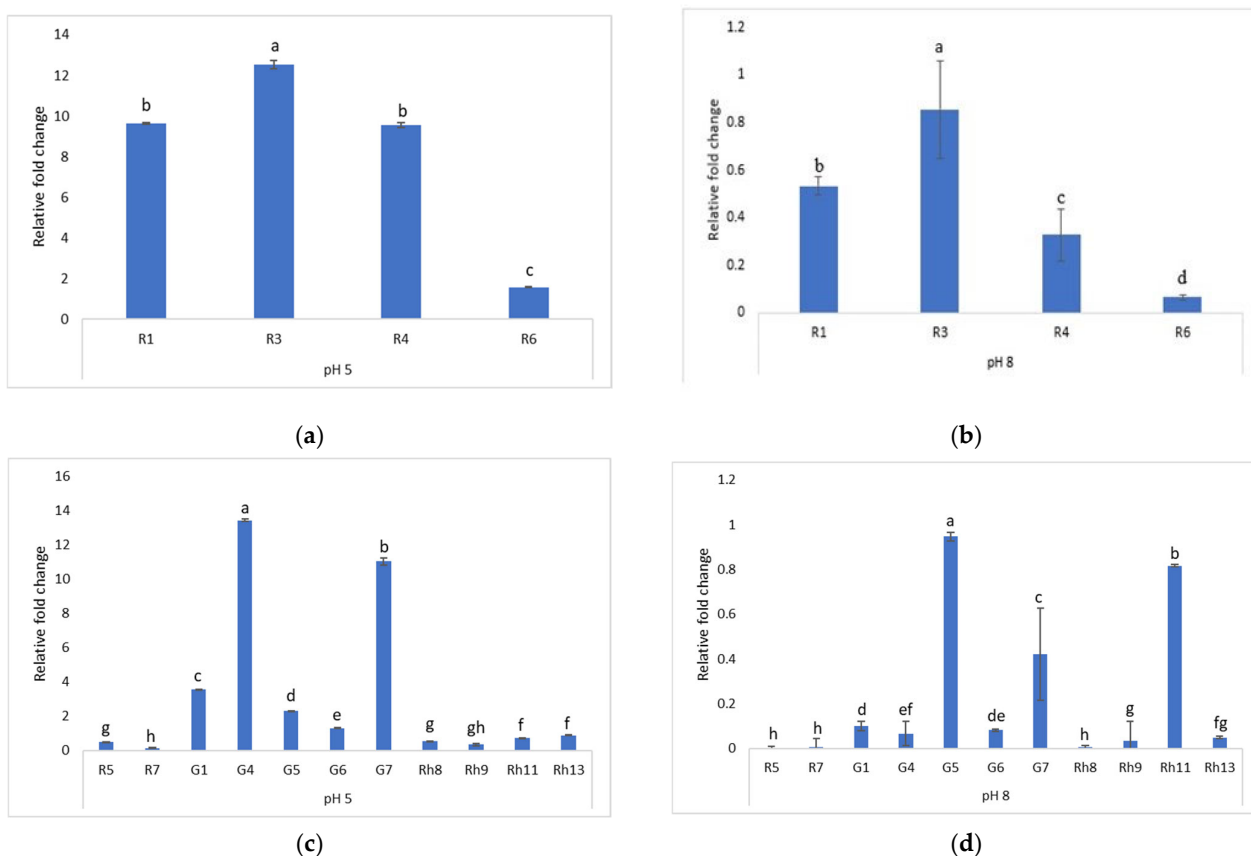


Figure 6. Xylanase gene expression of the selected 15 extracellular xylanase-producing lactic acid bacteria. (a) Fold of XylW xylanase gene expression of *W. confusa* at pH 5; (b) Fold of XylW xylanase gene expression of *W. confusa* at pH 8. (c) Fold of XylP xylanase gene expression of *P. pentosaceus* at pH 5. (d) Fold of XylP xylanase gene expression of *P. pentosaceus* at pH 8. The fold of xylanase gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method. Data are plotted as average values of relative fold change $\pm$ standard error. Bars represented with different lowercase letters are statistically significant at $p < 0.05$.

The pH of the growth medium plays a critical role in modulating xylanase synthesis and influencing various metabolic processes. The pH variable is of utmost importance on EXLAB since it has a direct impact on both microbial growth and enzyme biosynthesis [77,78], whereby the pH condition strongly affected the xylanase-encoding genes. Our results are in line with earlier reports related to the expression of the pH-dependent genes. For instance, the RT-qPCR analysis revealed a remarkable and parallel increase in mRNA accumulation for *xyn1* in *Humicola grisea* var. *thermoidea* at pH 8, in comparison to the acidic environment of pH 5.0 [36]. Moreover, Tanaka, Muguruma and Ohta [79] found higher expression levels of the *xynII* xylanase gene at pH 6.0 and 8.0, compared to pH 2.7, with fold increases of 8 and 22, respectively. Similarly, Lee et al. [80] demonstrated that the xylanase gene *xynT*, derived from *Bacillus alcalophilus* AX2000, which encoded an endo-beta-1,4 xylanase belonging to GH family 10, displayed its highest activity between pH 7 and 9.

Many microbes in nature experience wide swings in pH. Thus, a powerful pH homeostasis system and a regulatory mechanism are essential to meet the challenges of pH

change [81]. Moreover, the microorganisms face a significant barrier to growth and survival when they are unable to adapt to pH variations. Adaptation typically involves changes in gene expression, which can trigger alterations in metabolic pathways or the production of exported molecules with potential environmental effects. This process also occurred in regulating the synthesis of extracellular hydrolytic enzymes. Xylanase-encoding gene expression has been reported to be pH-regulated [36].

Despite the considerable studies that have been conducted on the control of xylanase gene expression in many other bacteria and fungi, there is a noticeable lack of studies focusing on LAB and their pH-dependent gene expression. Understanding the pH regulatory processes involved in LAB xylanase enzyme production is crucial for advancing biotechnology and effectively utilising renewable polymers. The examination of xylanase gene expression in LAB across different pH conditions provides valuable insights into the unique regulatory mechanisms involved, thereby adding valuable and essential information to the extracellular xylanase enzyme production and its applications.

3. Materials and Methods

3.1. Isolation of Lactic Acid Bacteria from Plant Sources

Fresh radish (*Raphanus sativus*), rhubarb (*Rheum rhabarbarum*) and gundelia (*Gundelia tournefortii*) were purchased from the wet market located in Erbil, Iraq and kept in a sanitised plastic bag. Subsequently, the entire plant was rinsed with sterile distilled water before being chopped into approximately 5 mm pieces. One gram of the chopped plant was suspended in 9 mL of sterile 0.85% (*w/v*) NaCl solution (Pharmacia, Uppsala, Sweden), followed by a 10-fold dilution and mixing well by vortex (Stuart Scientific, Stafford, UK) for 3 min. A volume of 100 μ L of suspension was evenly spread onto MRS agar plates (Neogen Co., Lansing, MI, USA). Subsequently, the cultured MRS plates were incubated under anaerobic conditions for 3 days at 30 °C. The single colonies from the cultured MRS plates were selected and cultured again on a new MRS agar plate [82]. Pure presumptive LAB isolates were maintained by culturing them on the surface of MRS slant agar that was tightly wrapped with parafilm and kept at 4 °C for 4–6 weeks. For long-term preservation, the pure presumptive LAB isolates were cultured in MRS broth (Neogen Co., Lansing, MI, USA), which consisted of 20% (*v/v*) glycerol (Merck, Darmstadt, Germany) and kept at –30 °C [83].

3.2. Screening of Extracellular Xylanase-Producing Lactic Acid Bacteria

The isolated presumptive LAB were determined for their capability of producing extracellular xylanase by culturing them on xylan-MRS agar supplemented with 2.5 g/L xylan and incubated at 30 °C for 48 h prior to embedding with a 0.5% (*w/v*) Congo red (BDH Chemicals Ltd., Poole, UK) solution prepared in 5% (*v/v*) ethanol (Sigma-Aldrich, Steinheim, Germany) for 15 min. Then, the plates were rinsed with 1 M NaCl (Pharmacia, Uppsala, Sweden) to remove the Congo red stain. The EXLAB was selected based on the presence of distinct, clear zones formed around the bacterial suspension well after the Congo red staining [84,85]. The diameter of the clear zone formed by each EXLAB-isolate was measured.

3.3. Specific Extracellular Xylanase Activity Determination

The specific extracellular xylanase activity was determined by quantifying the reducing sugars released from xylan substrate with 3,5-dinitrosalicylic acid (DNS) method developed by Miller [86].

3.3.1. Preparation of Extracellular Xylanase Enzymes

The procedure of Lee et al. [7] and Zabidi et al. [9] were employed to prepare the extracellular xylanase enzymes. The 15 EXLAB were grown in MRS broth for 24 h at 30 °C. The active EXLAB cultures (1 mL) were then collected by centrifugation for 15 min at $10,000 \times g$, 4 °C. The cell pellet was then rinsed once with a sterile 0.85% (*w/v*) NaCl solution (Pharmacia, Uppsala, Sweden), followed by resuspension of the washed cell pellet in 1 mL of sterile 0.85% (*w/v*) NaCl. The cell population of each EXLAB cell suspension was then adjusted with sterile 0.85% (*w/v*) NaCl to achieve an optical density (OD) of 0.1 at 600 nm, corresponding to a cell population of approximately 10^9 CFU/mL. The working cultures of EXLAB isolates were prepared by introducing 10% (*v/w*) of an OD-adjusted active inoculum (containing approximately 10^9 CFU/mL viable cells) into MRS broth and incubating at 30 °C for 24 h. The CFS was collected by centrifugation for 15 min at $10,000 \times g$, 4 °C, followed by filtration using a cellulose acetate membrane filter (Sartorius Minisart, 0.22 µm, Göttingen, Germany) as described by Lee et al. [7]. The clear-filtered supernatant was considered a crude enzyme used to determine the specific extracellular xylanase activity at pH 5, 6.5 and 8, respectively. The filtered CFS was kept at −20 °C until xylanase enzyme assays were performed.

3.3.2. Effect of pH on Extracellular Xylanase Activity

The specific extracellular xylanase enzyme activities of the 15 EXLAB isolates were determined according to the method described by Zabidi et al. [9] at pH 5.0 with a 0.1 M sodium acetate buffer solution, pH 6.5 with a 0.1 M sodium phosphate buffer solution and pH 8.0 with a 0.1 M Tris-hydrochloric acid buffer solution. Birchwood xylan (Sigma, St. Louis, MO, USA) solution of 1% (*w/v*) was used as substrate. The xylan solution was prepared using the corresponding buffer solutions of pH 5, 6.5, and 8, respectively. The extracellular xylanase enzyme assay mixtures included: (1) enzyme blank (EB) as positive control, which contained the enzyme and buffer but no substrate; (2) substrate blank (SB) as negative control, which contained the substrate and buffer but no enzyme; and (3) reaction mixture (RM), which contained the enzyme, substrate and buffer, respectively. All assay mixtures were incubated at 37 °C for 1 h.

The reduced sugar concentrations produced from xylan substrate by the extracellular xylanase were determined using the modified DNS method of Miller [86], as described by Zabidi et al. [9]. At the end of the 1 h incubation, 100 µL of DNS reagent and 10 µL of sodium hydroxide (0.1 M NaOH) were added to the 100 µL assay mixture solution. Subsequently, the assay mixture solution was boiled for 5 min prior to adding 800 µL of deionised water (dH₂O) and left at room temperature for 20 min. The absorbance (Abs) at 540 nm was measured using a UV–visible spectrophotometer (Genesys 20, Thermo Fisher Scientific, Waltham, MA, USA). To determine the extracellular xylanase activity, xylose was employed as a standard reference.

The reference solution for Abs determination (RA) consisted of a mixture of DNS and NaOH. The reference absorbance was adjusted by subtracting the net absorbance derived from the enzyme blank, substrate blank and reaction assay mixture. The net Abs value was calculated using the following Equation (1).

$$\text{Net Abs} = (\text{RM} - \text{RA}) - (\text{SB} - \text{RA}) - (\text{EB} - \text{RA}) \quad (1)$$

where RM is the absorbance of the reaction mixture, SB is the absorbance of the substrate blank, EB is the absorbance of the enzyme blank and RA is the absorbance of the reference solution.

3.3.3. Protein Concentration Determination

The protein concentration of filtered CFS was determined by the modified Lowry method, as outlined by Miller [87]. Bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA) served as a standard reference. The term “one unit of specific enzyme activity (U/mg)” refers to the quantity of reducing sugar (μg) released in one minute of reaction time by 1 mg of protein under the specified assay conditions.

3.4. Phenotypic Identification of Extracellular Xylanase-Producing Lactic Acid Bacteria

Morphological and biochemical investigations were conducted for phenotypic identification. The presumptive xylanase-producing LAB isolate was cultured on MRS agar to examine its morphological features. The colony's shapes, edges, height and size were evaluated by careful observation. To investigate the morphology, the EXLAB underwent Gram's staining. Following the staining process, the presumptive EXLAB morphology was observed under an oil immersion objective lens at a magnification of $100\times$ to assess its form, size and reaction to the Gram stain. To assess catalase activity, a single colony of EXLAB was mixed with 3% (*v/v*) hydrogen peroxide (BDH, Merck, Poole, UK). The absence of bubbles within 15 s is an indication of the negative result [88]. The EXLAB were tested for their capabilities of producing lactic acid by culturing on MRS agar supplemented with 20 g/L calcium carbonate (Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated at 30 °C for 48 h. A soluble calcium around the EXLAB was considered a positive result [89]. Furthermore, EXLAB were further characterised for their growth at different temperatures by inoculating MRS broth with a 5% (*v/v*) overnight culture and incubating 3 days at 10, 25, 30, 37 and 45 °C, respectively [82]. The growth at different salt concentrations was also investigated in MRS broth containing 3.0, 4.5 and 6.5% (*w/v*) NaCl at 30 °C for 72 h [82].

3.5. Genotypic Identification of Extracellular Xylanase-Producing Lactic Acid Bacteria

The selected 15 EXLAB were identified using a full-length 16S rDNA sequence with universal primers 27F and 1492R.

3.5.1. Genomic DNA Extraction and Amplification

DNA was extracted from the 15 selected EXLAB isolates according to the manufacturer's instructions of Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., New Taipei, Taiwan). The integrity and purity of the extracted genomic DNA were evaluated using a NanoDrop (Thermo Scientific, Waltham, MA, USA). To amplify the 16S rDNA fragment, universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTACGACTT-3') (Macrogen, Seoul, Republic of Korea) were employed. After PCR amplification, a 15% (*w/v*) agarose gel electrophoresis was used to analyse the amplicon. The amplicon of the 15 EXLAB isolates was subjected to DNA sequencing by Macrogen Corporation in Korea. The retrieved 16S rDNA sequences were then compared with the consensus sequences deposited at the GenBank database of the National Centre for Biotechnology Information (NCBI). The alignment of the 16S rDNA sequence was evaluated with the NCBI BLAST tool for the EXLAB genotypic identification.

3.5.2. Phylogenetic Tree Analysis

Phylogenetic analysis was performed using the full 16S rDNA sequences of EXLAB. The 16S rDNA sequences were aligned for the evolutionary history studies using the MEGA X programme [90]. The initial trees for the heuristic search were obtained by applying the Neighbour-Join and BioNJ algorithms on a matrix of pairwise distances evaluated using the Maximum Composite Likelihood (MCL) model [91].

3.6. Amplification of XylW and XylP Xylanase Genes

The extracted genomic DNA of EXLAB were used to amplify the XylW and XylP genes from the *W. confusa* and *P. pentosaceus* strains, respectively. The XylW fragment was amplified using the XylW forward (F) primer: GGCTTCTTCAAGTGGTCAGC and reverse (R) primer: CGCCTTCTTCTTCATCCTTG. Meanwhile, the XylP fragment was amplified using the XylP F primer: TTTACCTGCCGTTACCCAAG and R primer: TGGGTGTTTTTG-GTTTGACA. The thermal cycles of amplification involved one cycle of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 57 °C for 20 s and extension at 72 °C for 20 s. Following the completion of the PCR cycles, a final extension step at 72 °C for 6 min was conducted. The amplified XylW and XylP genes were analysed by 15% (*w/v*) agarose gel electrophoresis. The size of the amplicon was determined by using a 100 bp DNA ladder as a reference.

3.7. Expression of XylW and XylP Genes by RT-qPCR

The expression of the XylW and XylP genes was analysed using RT-qPCR. The primers designed for this study are listed in Table 3. The sequences available at NCBI were used to design the primers with the primer3plus software (version: 3.2.0).

Table 3. Xylanase genes and the respective designed primers.

Primer	Primer Sequence (5-3)	Product Size (bp)	Annealing Temp (°C)	Strain, Accession Number and Region
XylW F	GGCTTCTTCAAGTGGTCAGC	196	57	<i>Weissella confusa</i> strain VTT E-133279 Accession number (CP027563.1) Region 1..1167
XylW R	CGCCTTCTTCTTCATCCTTG			
XylP F	TTTACCTGCCGTTACCCAAG	189	57	<i>Pediococcus acidilactici</i> strain SRCM101189 Accession number (CP021529.1) Region 1..897
XylP R	TGGGTGTTTTTGTTTGACA			
recAW F	TGACTCAACTGTCCGTTTGC	156	58	<i>Weissella confusa</i> strain LM1 Accession number CP080582.1 region: 899577..900722
recAW R	GTCCACCAGGTGTCGTTTCT			
recAP F	GCAGTTGCTGAAGTGCAAAA	167	57	<i>Pediococcus pentosaceus</i> strain MR001 Accession number CP047081.1 Region 1246887..1247945
recAP R	GATACCAAAGCATCGGCAAT			

Notes: recAW and recAP are housekeeping genes, whereas XylW and XylP are xylanase genes.

3.7.1. RNA Extraction and Quantification

The total RNA from the 15 EXLAB that grew at pH 5, 6.5, and 8 was extracted with TRIzol® Plus RNA Purification Kit (Thermo Fisher Scientific, Waltham, USA), following the manufacturer's instructions. Briefly, EXLAB culture was centrifuged for 1 min at 13,000× *g*. The supernatant was discarded. Then, 1 mL of TRIzol was added to lyse the EXLAB cell pellet at room temperature for 5 min. A volume of 0.2 mL of chloroform was then added to the lysate and incubated at room temperature for 2–3 min, prior to centrifugation for 15 min at 12,000× *g* to separate the mixture into a lower organic phase, an interphase and a colourless upper aqueous phase. The aqueous phase containing the RNA was transferred into a new tube. A volume of 200 µL of isopropanol was then added to the aqueous phase, followed by centrifugation for 15 s at 12,000× *g*. The total RNA was precipitated on the filter of the spin column. The supernatant was then discarded. The precipitated total RNA was resuspended in 0.5 mL of washing buffer I, followed by centrifugation for 15 s at 12,000× *g*, and the supernatant was discarded. The total RNA in the column was resuspended again with 0.5 mL of washing buffer II, followed by centrifugation for 15 s at

12,000× *g*, and the supernatant was discarded. Next, 75 µL RNase-free water was added and centrifuged at 12,000× *g* for 2 min to elute RNA. The 260/280 adsorption ratio of more than 2.00 was considered optimal purity of the extracted RNA. Finally, the purified total RNA was stored at −20 °C.

3.7.2. Reverse Transcription and Pre-Amplification of cDNA

Purified total RNA was converted to complementary DNA (cDNA) using a cDNA synthesis kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The reverse transcription reactions contained 1 µg of purified total RNA and 2 µL of hexamer primer. Nuclease-free water was then added to reach a total volume of 20 µL. The PCR was performed with the following amplification conditions: 37 °C for 10 min, followed by 42 °C for 1 h and 95 °C for 5–10 min in a single cycle. Then, RT-qPCR was performed to amplify either the XylW or XylP xylanase gene from the cDNA, using their respective primers. RecAW and recAP were employed as the housekeeping genes to standardise the expression of both xylanase genes. The qPCR mixture was made in an AccuPower® RT PreMix tube, which comprises 1 µL of forward and reverse primers, 2 µL of template cDNA and the volume was brought to 20 µL with DNase-free distilled water and mixed well. The following Real-Time PCR programme was performed: one cycle at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 45 s and 72 °C for 60 s. The relative changes in mRNA expression levels were determined using a comparative threshold cycle (CT) method ($2^{-\Delta\Delta C_t}$) as described by Livak and Schmittgen [92]. The Livak equation was used to evaluate the fold expression of the target xylanase genes against housekeeping genes, as described in Equation (2).

$$\text{Relative Fold of gene expression} = (2^{-\Delta\Delta C_t}) \quad (2)$$

$$\Delta\Delta C_t = \Delta C_t \text{ of the treated sample} - \Delta C_t \text{ of the untreated sample}$$

ΔC_t of treated sample:

$$\Delta C_t \text{ of treated sample} = C_t \text{ of interest gene} - C_t \text{ of housekeeping gene}$$

ΔC_t of untreated sample:

$$\Delta C_t \text{ of untreated sample} = C_t \text{ of interest gene} - C_t \text{ of housekeeping gene}$$

where C_t : Represents the sample's cycle threshold (C_t).

Delta (Δ): to describe the difference between two numbers.

3.8. Statistical Analysis

The experiments were performed using biological replicates. The values were reported as the mean $\pm$ standard error of the mean (SEM). The statistical analyses were conducted using R Studio software (version 2023.09.1+494).

4. Conclusions

LAB have been reported for their ability to produce versatile extracellular enzymes. They have attracted vast attention for several decades, owing to their GRAS status and involvement in many biotechnological and industrial applications. Therefore, an attempt was made to isolate EXLAB from various plant sources in this study. We have successfully identified and characterised 15 EXLAB isolated from radish, rhubarb and gundelia, respectively, using semi-qualitative and quantitative assay methods to determine the extracellular xylanase activity. The EXLAB isolates of R1, R3, R4 and R6 were identified as *W. confusa*, and the EXLAB isolates of R5, R7, G1, G4, G5, G6, G7, Rh8, Rh9, Rh11 and Rh13 were iden-

tified as *P. pentosaceus*. The 15 selected EXLAB isolates demonstrated extracellular xylanase activity over a broad pH range, spanning from acidic pH 5 to alkaline pH 8. Nevertheless, *P. pentosaceus* G4 displayed the most significant extracellular xylanase activity (0.88 U/mg) at pH 5. The presence of XylW or XylP xylanase genes was verified for *W. confusa* strains and *P. pentosaceus* strains, respectively. The investigation of xylanase gene expression in the 15 EXLAB strains revealed that the expression of the xylanase genes occurred over a broad pH range, from acidic to alkaline, which was in line with the displayed specific extracellular xylanase activity of the studied EXLAB strains, inferring the potential adaptation of EXLAB to various environmental pH conditions. Notably, the highest fold of extracellular xylanase gene expression was observed in *P. pentosaceus* G4, with a fold of 13.45 at pH 5, suggesting a pH-dependent regulatory mechanism involved in the xylanase gene expression. This reflects the metabolic traits of this strain and highlights its acidophilic nature. The robust xylanase gene expression, with the specific extracellular xylanase activity presented in this study, has revealed the vast potential of *P. pentosaceus* G4 as a versatile and eco-friendly extracellular xylanase producer for various xylanase enzymatic processes in respective industries and for environmentally friendly solutions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal15100990/s1>, Supplementary Figure S1: Agarose gel electrophoresis analysis of the 16S rDNA amplified from the selected EXLAB. M: 100 bp ladder marker, Lanes 1–15 resemble 1500 bp PCR products, 1: R1, 2: R3, 3: 4, 4: R5, 5: R6, 6: R7, 7: G1, 8: G4, 9: G5, 10: G6, 11: G7, 12: Rh8, 13: Rh9, 14: Rh11, 15: Rh13; Supplementary Figure S2: Agarose gel electrophoresis analysis of the amplified xylanase genes of the selected EXLAB. (a) Amplified 196 bp XylW.

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