Regular Article
pISSN: 2288-9744, eISSN: 2288-9752
Journal of Forest and Environmental Science
Vol. 40, No. 2, pp. 118-122, June, 2024
https://doi.org/10.7747/JFES.2024.40.2.118

JFES
Journal of Forest and Environmental Science

Preparation of Microbial Media based on Hardwood Fallen Leaves

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Abstract

This study reported the novel use of fallen leaf extract as a microbial culture media for the first time. Extract from hardwood fallen leaves (HLE) was prepared under high temperature and pressure conditions and then supplemented with specific nutrients. The growth of four industrially significant prokaryotes on the HLE-based media was measured and compared with that on enriched media (Luria-Bertani, LB). Notably, supplementing HLE with only 0.5 g of yeast extract and 1 g tryptone per liter showed a similar growth rate of *Pseudomonas chlororaphis* compared to standard LB media. Overall, the HLE media developed in this study offers a sustainable and cost-effective approach to microbial media production, capitalizing on the valorization of forest waste.

Key Words: microbial media, fallen leaves, *Pseduomonas chlororaphis*, forest waste valorization

Introduction

Unused forest biomass refers to materials in forest residues (barks, branches, leaves, sawdust, and others) unutilized during forest management and timber harvesting due to their low economic value and limited industrial applications. Most unused forest biomass is processed into wood chips and pellets, which are then used for producing renewable energy (Braghiroli and Passarini 2020), however, fallen leaves are not considered a typical raw material for wood chips and pellets. In the ecosystem, fallen leaves are naturally decomposed and contribute to soil fertility as compost, however, they can also accelerate wildfire (Kim et al. 2017). In urban areas, these leaves are a primary cause of sewer blockages, leading to floods during the rainy season (Palla et al. 2018).

In Korea, over 8.5 million street trees produce approximately 800,000 tons of fallen leaves annually (Choi et al. 2020), and these leaves are collected as municipal waste. They are typically disposed of through landfilling or incineration, which requires significant space and incurs costs (Kim and Sung 2020). Therefore, the utilization of fallen leaves as high-value-added concepts has been investigated: Composting is a prevalent approach, as fallen leaves contain nutrients such as nitrogen, phosphorus, and potassium which are crucial composite elements (Ayilara et al. 2020). In addition, fallen leaves have been used as adsorbents for various toxic dyes representing potential use in bioremediation (Bulgariu et al. 2019). More recently, Yang and colleagues have demonstrated that biohydrogen can be produced from fallen leaves by co-fermentation of sewage sludge and fermentation residue (Yang et al. 2019; Yang

Received: April 18, 2024. Revised: May 20, 2024. Accepted: May 20, 2024.

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and Wang 2021).

In this study, we explored the novel use of fallen leaves as a microbial growth medium. We prepared an extract from hardwood fallen leaves (HLE) via autoclaving and evaluated the its efficacy in supporting the growth of four industrially important prokaryotic microorganisms. By supplementing specific nutrients to HLE, we developed a microbial medium that surpassed the commonly used Luria-Bertani (LB) medium in fostering cell growth.

Materials and Methods

Media preparation using hardwood fallen leaves (HLE)

The mixture of hardwood fallen leaves was collected from the Kangwon National University campus (Chuncheon, Korea). The major constituents of the mixture were identified *Acer buergerianum*, *A. triflorum*, and *Quercus dentata*. To make hardwood fallen leaves extract (HLE), 150 g of the mixture was added to the 3 L distilled water and autoclaved at 121°C for 60 min. The extract was then filtered through 0.45 μm membrane filter (Merck, Rahway, NJ, USA) to remove solid content. The chemical and nutritional properties of the HLE were analyzed by ISA Research Institute (Uiwang, Korea) and AT Analysis Technology (Incheon, Korea), respectively. The HLE was used as a liquid formulation media, and to prepare a solid media using the HLE, 20 g of Bacto Agar (Difco BD, Franklin Lakes, NJ, USA) was added to the 1 L of the HLE. Luria-Bertani (LB) media was purchased from Difco BD and used as an enriched media. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Microbial growth in HLE media

Microbial growth in the HLE media was investigated using four industrially significant bacteria purchased from Korean Collection for Type Cultures (KCTC, Jeongeup, Korea): *Bacillus subtilis* (KCTC #1027), *Escherichia coli* (KCTC #1116), *Pseudomonas chlororaphis* (KCTC #52142), and *Pseudomonas putida* (KCTC #1751). The bacteria were activated on LB plate, and individual colonies were transferred to the HLE plate. The plates were then incubated at 30°C for 2 days. To compare cell growth between HLE and LB media, *P. chlororaphis* was cultured in 3 mL LB broth at 30°C and 180 rpm for 16 h (seed culture), and 500 μL seed culture was inoculated into the 10 mL HLE and LB media and cultured under the same condition for 24 h. Cell growth was analyzed by measuring optical density at 600 nm (OD_{A600}). To enhance the microbial growth of HLE media, various concentrations of yeast extract and tryptone, as detailed in Table 1, were added, and the growth rate of *P. chlororaphis* was measured as described above.

Statistical analysis

Statistical analysis of the microbial growth comparison was conducted using unpaired t-test and one-way analysis of variance (ANOVA) followed by Turkey's multiple comparisons, using GraphPad Prism 10 software.

Results and Discussion

Component analysis of the HLE

To evaluate the potential use of HLE for microbial media, the chemical and nutritional composition of the prepared HLE was analyzed and compared to the standard

Table 1. Composition of microbial media used in this study

	LB	HLE	HLE+0.5×	HLE+0.25×	HLE+0.1×	HLE+0.05×
Yeast extract	5	0	2.5	1.25	0.5	0.25
Tryptone	10	0	5	2.5	1	0.5
NaCl	5	5	5	5	5	5
HL	-	45	45	45	45	45

Unit: g/L.
HLE, hardwood fallen leaves extract; HL, hardwood fallen leaves.

LB medium. LB media consists of yeast extract and peptone, which are rich in various biological nutrients including nucleotides, amino acids, sugars, and various trace elements (Tao et al. 2023). As described in Table 2, compared to LB, the prepared HLE presents notably lower quantities of most listed components except fats, sulfur, and boron. In particular, the concentration of salt (sodium chloride), which is essential for microbial media to maintain osmotic pressure, was found in HLE significantly lower than LB implying the HLE may not be suitable for microbial cultivation. To address this concern, 10 g/L of sterilized sodium chloride was added to the HLE before conducting microbial growth assays.

Prokaryotic growth on HLE

Microbial growth on HLE was conducted using four industrially significant prokaryotes: *B. subtilis* is well-known gram-positive bacteria employed for the production of industrial enzymes such as amylases and proteases, and is involved in the production of fermented foods and biopesticides (Lengai and Muthomi 2018; Su et al. 2020). *E. coli* is extensively used in biotechnology and genetics as a model organism and is particularly favored for recombinant protein production (Theisen and Liao 2017). *P. chlororaphis* and *P. putida* have been applied in agriculture as a biological control agent due to their production of antifungal substances (Shtark et al. 2003). In addition, they have been recently highlighted for their bioremediation potential (Weimer et al. 2020; Vélez et al. 2021).

Upon streaking the four microbes on HLE plates, only two *Pseudomonas* species exhibited growth, in contrast to those on LB (Fig. 1). As described above, *Pseudomonas* species are regarded as workhorses of bioremediation indicating capable of decomposing toxic pollutants such as phenolic compounds and heavy metals. In this respect, the HLE may contain uncharacterized toxic compounds that are not characterized in this study affecting the growth inhibition of *B. subtilis* and *E. coli*. Therefore, we chose one

Table 2. Composition analysis of HLE and LB

Components	HLE	LB	Note
kcal/L	32	9.25	↑
Carbohydrate (g/L)	n/d	-	
Sugar (g/L)	n/d	0.08	↓
Fat (g/L)	3.2	0.045	↑
Trans fat (g/L)	n/d	-	
Saturated fatty acid (g/L)	0.2	-	↑
Cholesterol (g/L)	n/d	-	
Protein (g/L)	0.8	1.195	↓
Potassium (g/L)	0.1832	200	↓
Calcium (g/L)	0.1681	5.25	↓
Magnesium (g/L)	0.0366	9.65	↓
Sodium (g/L)	0.0094	4,307.5	↓
Nitrate (g/L)	0.0280	-	↑
Phosphorus (g/L)	0.0137	5.2	↓
Sulfur (g/L)	0.1502	-	↑
Chlorine (g/L)	0.7090	6,073.0	↓
Boron (g/L)	0.0013	-	↑
Zinc (g/L)	0.0001	0.2095	↓
Copper (g/L)	n/d	0.0123	↓
Manganese (g/L)	0.0056	0.0131	↓
Iron (g/L)	0.00015	0.2020	↓
Molybdenum (g/L)	n/d	-	
pH	5.25	7	

n/d, not detected.
The arrows indicate higher (↑) and lower (↓) concentrations relative to LB.

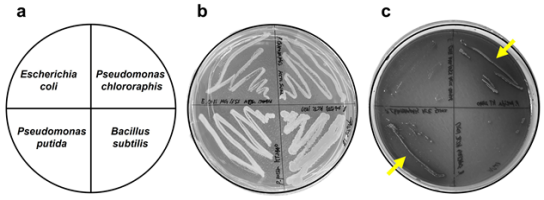

Fig. 1. Streak plate method verifying the growth of four bacteria. Schematic representation of the quadrant streak plate (a). Four prokaryote growth patterns on LB and HLE were represented in (b) and (c), respectively. In the panel (c), distinct growth patterns were indicated by arrows.

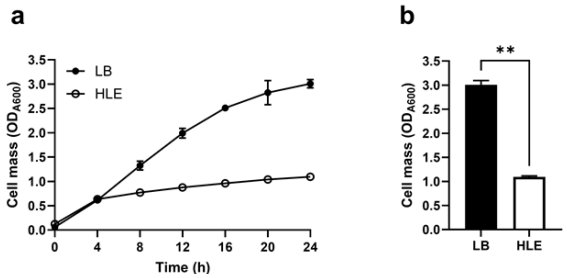

Fig. 2. Growth comparison of *P. chlororaphis* in LB and HLE media. The cell mass of *P. chlororaphis* in LB and HLE during 24 h incubation was plotted on (a). Panel (b) presents a statistical analysis of the final cell mass in both media. Statistical significance was and represented as the number of asterisks. *p<0.05, **p<0.01, ***p<0.001, ns, not significant.

Pseudomonas species (*P. chlororaphis*) for further experiments.

The growth patterns of *P. chlororaphis* in HLE broth were compared to those in LB broth. As showed in Fig. 2a, the optical density at 600 nm reached approximately 1 in HLE media, whereas it exceeded 3 in LB media. Notably, the growth curve in HLE indicated a rate-limiting pattern after 4 h, with the final cell mass significantly lower than measured in LB culture (Fig. 2b). This discrepancy can be attributed to two primary factors: Firstly, as previously mentioned, uncharacterized toxic compounds in HLE may inhibit the cell growth of *P. chlororaphis*. Secondly, a lower concentration of nutrients, especially nitrogen sources, may contribute to restricted cell growth. To verify the latter hypothesis, we supplemented the HLE with additional nutrients.

Supplementation of HLE media

To enhance the growth of *P. chlororaphis* in HLE media, nitrogen sources were added as specified in Table 1.

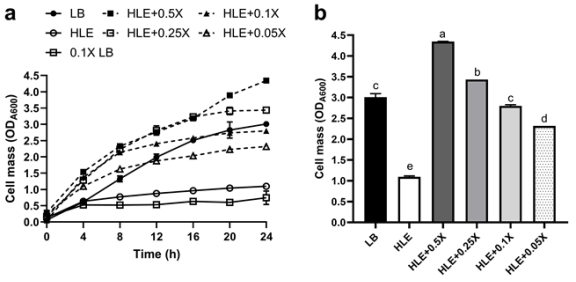

Fig. 3. Growth comparison of *P. chlororaphis* in supplemented HLE media. Panel (a) plots the cell mass of *P. chlororaphis* cultured in LB and supplemented HLE media. Final cell mass after 24 h of growth in different media was statistically analyzed (b).

Subsequent growth comparisons revealed a dose-dependent recovery in cell growth with nitrogen supplementation to the HLE media, as depicted in Fig 3a. This recovery suggests that the growth limitations in HLE media were attributed to insufficient nitrogen sources. A statistical analysis of the final cell mass identified five distinct groups. Notably, the HLE+0.1× formulation was categorized in the same group as the standard LB media, facilitating a significant cost reduction. The cost for achieving a cell mass with an OD_{A600} of 1 for each media was determined based on Table 1. The cost for HLE+0.1× media was computed at 222.6 KRW, significantly lower than the 1,967.6 KRW required for standard LB. This calculation reveals that culturing *P. chlororaphis* in HLE+0.1× media is approximately 8.8 times more cost-effective than using standard LB (Table 3). To develop this study, we consider adding urea as a nitrogen source replacing the yeast extract and tryptone enabling a more cost-saving strategy (Jahns 1992). In addition, the cultivation of industrially useful microbes in HLE from specific tree species should be verified for the general use of HLE for broader applications. Further, a valuable product such as biopolymer that can be applied in bioplastic industry will be produced by *P. chlororaphis* in the HLE-based media.

Conclusions

The study firstly reported that hardwood fallen leaves extract (HLE) could be a cost-effective alternative to LB medium for culturing industrially significant bacteria when supplemented with nitrogen and sodium chloride. In HLE

Table 3. Cost calculation of HLE media

	LB	HLE	HLE+0.5×	HLE+0.25×	HLE+0.1×	HLE+0.05×
Yeast extract	1,180	0	590	295	118	59
Tryptone	4,708	0	2,354	1,177	470.8	235.4
NaCl	34.4	34.4	34.4	34.4	34.4	34.4
HL	0	0	0	0	0	0
Total	5,922.4	34.4	2,978.4	1,506.4	623.2	328.8
Cell mass (OD_{A600})	3.01±0.06	1.10±0.02	4.35±0.01	3.44±0.04	2.80±0.02	2.32±0.01
Cost for OD_{A600}=1	1,967.6	31.3	684.7	437.9	222.6	141.7

Unit: KRW.
Labor and utility costs are not taken into consideration.

media, notably, the modified HLE+0.1× medium matched LB's effectiveness in supporting bacterial growth and was approximately 8.8 times more economical, indicating significant potential for industrial microbial applications.

Acknowledgements

This study was supported by 2023 Research Grant from Kangwon National University.

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