IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 18, Issue 3 Ser. 1 (March 2024), PP 07-13
www.iosrjournals.org

Isolation And Characterization Of Lignin-Degrading Bacteria From Cow dung

Arshid T, Amal K Aravind*, Vishruth Vijay, M. Abdul Rasheed, Priyank Bhutiya

Environment Management, Gujarat Energy Research And Management Institute, Pandit Deendayal Energy University Campus, Raysan, Gandhinagar 382007, Gujarat, India.

Abstract

Background:*Lignin, a structurally complex polymer inherent to plant cell walls, presents a formidable obstacle due to its recalcitrant nature. Microbial degradation of lignin offers a promising avenue for its sustainable utilization across various industries. In this investigation, we endeavored to isolate and characterize lignin-degrading bacteria fromcowdung, recognized for its rich microbial diversity.*

Materials and Methods: *In this study we have isolated lignin degrading bacteria from the cow-dung sample. It was screened using two different types of media. One was with Lignin 1 g in 100 ml of MSM with 1 g methyl blue and 0.3 ml of trace elements and another was with Medium of 100 ml MSM with 0.500 g alkali lignin and 0.25 g methyl blue and 0.3 ml of trace elements. The lignin degrading efficiency was measured using a modified version of Klanson lignin analysis.The isolated bacterial species were characterized using MALDI.*

Results:*We have isolated a total of 5 bacteria from the bovine faeces. The lignin-degrading proficiency of the selected isolates was assessed through both qualitative and quantitative methodologies, including assays measuring ligninolytic enzyme activities and degradation efficiencies. The results from the lignin degradation assays shows that the bacteria labelled LDB/2023/01 degrades higher amount of lignin compared to the other isolated bacteria. All other species shows negative result of degradation. We have identified the isolated bacteria as Acinetobacter schindleri through MALDI. Our findings illuminate the presence of a diverse array of bacterial taxa proficient in the efficient degradation of lignin constituents present in bovine faeces.*

Key Word*: Lignin degradation, Bacterial isolation, Bovine faeces, MALDI, Bioremediation.*

Date of Submission: 16-03-2024 Date of Acceptance: 26-03-2024

I.Introduction

Lignin, a complex and recalcitrant biopolymer, is a major component of lignocellulosic biomass. It contributes to the rigidity and structural integrity of plants. However, the presence of lignin poses a challenge in the efficient utilization of biomass for various applications, including bioenergy production. Lignin degradation is a crucial step in accessing the fermentable sugars present in biomass, which can then be used for the production of biofuels and other value-added products.

Lignin is formed through the polymerization of phenolic compounds, such as coniferyl, sinapyl, and p-coumaryl alcohols, resulting in a complex and highly resistant structure (Boerjan et al., 2003). This intricate network of linkages and cross-links makes lignin difficult to break down, limiting the efficient conversion of lignocellulosic biomass into useful products.

Lignin is composed of three main types of monolignols: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These monolignols undergo oxidative coupling reactions to form different structural units within the lignin polymer. The most abundant structural units found in lignin are guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units, which arise from coniferyl, sinapyl, and p-coumaryl alcohols, respectively (Boerjan et al., 2003).

The structure of lignin is highly complex and heterogeneous, varying depending on the plant species and tissue type. It consists of an irregular three-dimensional network of interconnected aromatic units, linked together by various types of chemical bonds. The primary linkages within lignin are ether linkages (β-O-4), which connect two aromatic units, followed by carbon-carbon linkages (β-β, 4-O-5, 5-5, etc.) and carbon-oxygen linkages (α-O-4, β-5, etc.).

The abundance and distribution of different linkages in lignin contribute to its recalcitrant nature. The presence of cross-links, such as β-β and β-5 linkages, and the formation of condensed structures further enhance its resistance to degradation. These complex linkages and cross-links create a barrier, preventing the efficient

access of lignocellulosic biomass to enzymes or other biological catalysts that can break down the lignin structure.

Efforts to overcome the challenges associated with lignin degradation and valorisation have been ongoing. Various methods have been explored, including chemical treatments, thermal processes, and biological approaches. Chemical methods involve pre-treatments to modify the structure of lignin, while thermal processes use heat and pressure to depolymerize lignin into smaller fragments. Biological approaches, such as enzymatic and microbial degradation, employ natural or engineered systems to break down lignin.

Understanding the detailed structure of lignin is crucial for developing effective strategies to overcome its recalcitrance and harness its potential as a valuable resource. By unraveling the intricate arrangement of linkages and cross-links, scientists can design targeted approaches to selectively degrade lignin while preserving the desired properties of lignocellulosic biomass. This knowledge can contribute to the development of efficient lignin valorization processes and the sustainable production of biofuels, chemicals, and materials from lignocellulosic feedstocks.

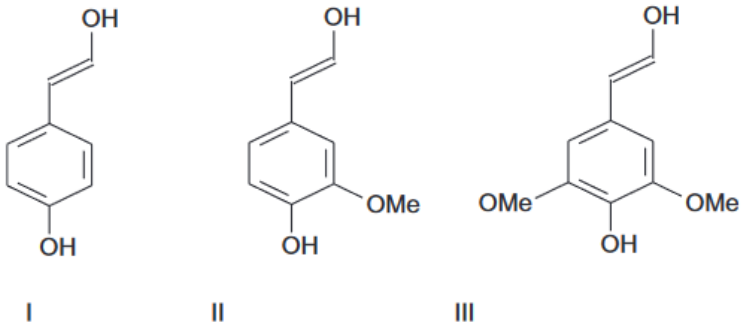

Figure 1.1*Lignin precursors. I: p-coumaryl alcohol (H); II: coniferyl alcohol (G); III: sinapyl alcohol (S).*

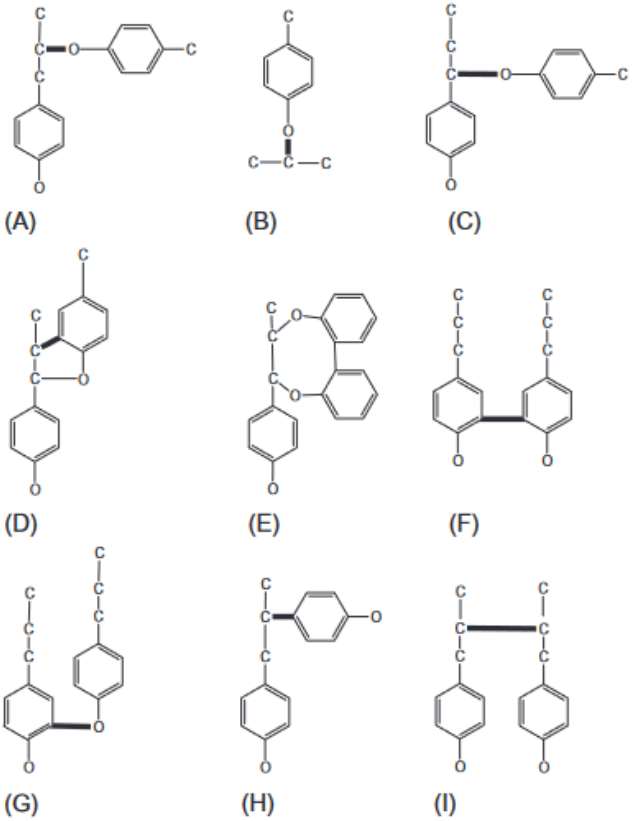

Figure 1.2*The key bond types between the phenylpropane units in MWL the linkages in β-O-4 (A),glycerol-2-aryl ether (B), α-O-4 (C), β-5 (D), dibenzodioxocin (E), 5-5 (F), 4-O-5 (G), β-1 (H), β-β (I).*

II. Material And Methods

Sample collection

Fresh cow dung samples were collected from a local dairy farm. Care was taken to collect samples that were representative of the overall cow dung composition. The samples were placed in sterile polyethylene bags and transported to the laboratory while maintaining minimal temperature within two hours of collection to minimize any potential changes in microbial composition (Jones et al., 2020).

Isolation of Bacteria

Aseptic techniques were employed throughout the isolation process. A serial dilution series was prepared by transferring 1 gram of cow dung sample to 10 mL of distilled water in a sterile Eppendorf tube. The mixture was vortexed thoroughly to ensure proper dispersion of the sample and suspension of microorganisms (Garcia-Gonzalez et al., 2013). After a settling time of 5minutes, 1 mL of the supernatant was collected and transferred to a new sterile tube. Serial dilutions were performed by adding 1 mL of the supernatant to 9 mL of sterile distilled water, creating dilutions up to 10^-7 (Kumar et al., 2017).

Nutrient agar plates were prepared by pouring sterilized and cooled nutrient agar medium into sterile Petri dishes. Using a sterile spreader, 100 µL of the desired dilution (10^-7) was spread evenly over the surface of the nutrient agar plate (Alves et al., 2016). This technique was repeated for each dilution level on separate plates.

Incubation and Colony Development

The agar plates were incubated at 36°C for 24 hours to allow bacterial colonies to develop (Gupta et al., 2019). After the incubation period, the agar plates were examined for the presence of bacterial colonies with distinct morphological characteristics such as colour, texture, or shape.

Individual colonies that appeared distinct were transferred to fresh agar plates using sterile inoculation loops to obtain pure bacterial cultures.

The isolated colonies were screened by using methylene blue dye as an indicator, So the cultures were streaked onto different lignin concentration minimal salts medium (MSM) agar plates supplemented with methyl blue. The following types of plates were prepared:

The first type of plate was prepared by adding 1 gm of lignin and 1 gm of methyl blue into 100 ml of MSM. Additionally, 0.3 ml of trace elements solution, containing essential micronutrients, was incorporated into the medium. The medium was mixed thoroughly to ensure proper distribution of lignin and methyl blue throughout the agar.

For 0.5 g of alkali lignin and 0.25 g of methyl blue were added to 100 ml of MSM. Additionally, 0.3 ml of trace elements solution, containing essential micronutrients, was incorporated into the medium. The medium was mixed well to ensure even dispersion of alkali lignin and methyl blue in the agar. These plates provide different lignin concentrations for evaluating the bacterial growth and degradation ability in the presence of lignin.

Preparation of lignin degradation assays

To study the degradation of lignin by the bacterial strains we isolated, we utilized a modified minimal salts medium (MSM) broth. A total of 6 grams each of lignin and methyl blue were added to 600 ml of MSM broth, and the mixture was thoroughly mixed to ensure proper dispersion.The lignin and methyl blue-containing MSM broth was divided into 6 conical flasks, each with a capacity of 250 ml. Approximately 100 ml of the prepared broth was added to each flask.The conical flasks were securely covered, and then autoclaved to achieve sterilization.After the autoclaved broth had cooled down, 10 ml of pure cultures was aseptically transferred into 5 of the conical flasks containing the lignin broth. One flask was kept as a control without the addition of the pure culture. The inoculated conical flasks, along with the control flask, were placed in a rotary shaker set at 37 degrees Celsius and 150 rpm.Before placing the flasks in the shaker, the initial weights of the flasks were noted. These initial weights served as a baseline to determine the weight loss caused by lignin degradation by the bacterial strains. The flasks were allowed to incubate in the rotary shaker for 3 days, maintaining the specified temperature and shaking conditions. After 3 days of incubation, the flasks were taken out of the shaker, and the weights of the flasks were noted again. The flasks were placed back into the shaker for further incubation. On the 4th and 5th day of incubation, the weights of the flasks were checked again, noting any changes in weight. On the 5th day, the flasks were taken out for further analysis to evaluate the degradation products or any other desired measurements. By monitoring the weight loss of the flasks, the extent of lignin degradation can be estimated.

Determination of Lignin degradation assays

To determine the amount of lignin degraded by bacteria in the MSM medium a modified version of Klason lignin analysis is used here. Klason lignin analysis provides a quantitative measurement of the lignin content in biomass and can be used to assess lignin degradation by comparing the lignin content before and after a specific treatment or process.

The sample, in this case, the MSM medium containing lignin and the bacteria, is collected at a specific time point during the degradation experiment. The sample is subjected to acid hydrolysis using concentrated sulfuric acid (H2SO4) under specific conditions. This process breaks down the lignocellulosic matrix, hydrolyzing the cellulose and hemicellulose components while leaving behind the insoluble lignin residue. The acid hydrolysate is filtered to separate the insoluble lignin residue from the soluble components. The residue is washed with distilled water to remove any remaining acid and other impurities. The washed lignin residue is dried to a constant weight in an oven at a specific temperature. Once dried, the residue is weighed to determine the amount of lignin present. The amount of lignin degraded is determined by comparing the weight of the lignin residue in the sample with the initial weight of lignin added to the MSM medium.

III. Result And Discussion

Identification of bacterial strains

Individual colonies that appeared distinct and representative characteristics such as colour, texture, or shape were selected and transferred to fresh agar plates using sterile inoculation loops to obtain pure bacterial cultures. We have isolated a total of5 bacteria from the cow-dungLDB/2023/01,LDB/2023/02, LDB/2023/03, LDB/2023/04, LDB/2023/05 and in that 1 was found to be lignin degradingLDB/2023/01. The isolated bacteria after the preliminary characterisationwere sent to GBRC(Gujarat Biotechnology Research Centre) for the bacterial identification using MALDI. MALDI-TOF is becoming an important technique in microbiological diagnostics. Bacterial isolates are combined into a crystallised matrix substrate, which is then dried and applied to a glass or metal plate. Laser desorption is then used to ionise particles and separate them depending on mass. Pathogens can be identified using the resulting spectra, which are based on the libraries of reference organisms analysed (JL Jones 2015).

The isolated lignin degrading bacteria were identified as *Acinetobacter schindleri*by the MALDI results.

Name of the bacteria	Score value	NCBI identifier
Acinetobacter schindleri	1.97	108981

Fig. 3.1MALDI result with Name, Score value, and the NCBI identifier of the selected lignin degrading bacterial Isolates

Confirmation Test for Screening Ligninolytic Activity

According to Sasikumar et al., (2014), isolated colonies were screened by using methylene blue dye as an indicator. Pure cultures were streaked on methylene blue containing (0.25 g/100mL and 1.0g/100mL) containing alkaline lignin agar plates with different lignin concentrations(1.0g/100mL and 0.5g/100mL and) each. Plates were incubated at 30°c for 72 hr. The microbes posses' ligninolytic enzymes undergoes oxidation of indicator dye resulting in decolourisation of methylene blue. The decolourised colonies were selected as lignin degraders.

Fig. 3.2 Screening of the ligninolytic activity with 1g of lignin in the media with all 5 bacteria

In the analysis of 1 g lignin and 1 g methylene blue media among the five strains (LDB/2023/1, LDB/2023/2, LDB/2023/3, LDB/2023/4, and LDB/2023/5), an intriguing pattern emerged. LDB/2023/1 and

LDB/2023/2 exhibited clear colonies with a distinct transparent zone on top of their growth. This transparent zone indicated the presence of lignin-degrading activity, as it suggested the oxidation and subsequent decolorization of the methylene blue indicator dye. The clear colonies and pronounced transparent zones around LDB/2023/1 and LDB/2023/2 indicated that these two strains possessed robust ligninolytic enzymes capable of efficiently breaking down lignin. This finding was particularly noteworthy, as it suggested that LDB/2023/1 and LDB/2023/2 could hold great potential for lignin degradation applications, such as in the bioconversion of lignocellulosic biomass or environmental remediation processes.

In contrast, LDB/2023/3, LDB/2023/4, and LDB/2023/5 showed comparatively less growth and lacked the distinct transparent zone observed in LDB/2023/1 and LDB/2023/2. This outcome indicated a lower level of lignin-degrading activity in these strains. While some growth was observed, the absence of a transparent zone implied that these strains might possess weaker or less efficient ligninolytic enzymes. However, it is important to note that even though LDB/2023/3, LDB/2023/4, and LDB/2023/5 exhibited limited lignin degradation, they could still hold potential in other aspects of microbial biotechnology or environmental applications. Further investigation into their enzyme profiles and metabolic pathways could reveal additional insights into their unique capabilities and potential utility.

The results of this analysis highlight the diverse lignin-degrading abilities among the five strains tested. LDB/2023/1 and LDB/2023/2 showed promising lignin-degrading activity, as evidenced by the clear colonies and transparent zones observed on the methylene blue-containing media. On the other hand, LDB/2023/3, LDB/2023/4, and LDB/2023/5 demonstrated less impressive lignin-degrading capabilities.

Lignin degradation analysis

The study aimed to compare the lignin-degrading capabilities of the five different strains in lignin broth, along with a control sample without any strain. Each strain was inoculated into 100 ml of lignin broth, and the flasks were incubated in a shaker incubator at 37 degrees Celsius and 150 rpm for 7 days. The initial and final weights were recorded for each strain and the control to assess lignin degradation. The control flask exhibited a weight decrease of 2.633 grams, possibly attributed to natural abiotic lignin degradation in the lignin broth.

Significant differences in lignin degradation were observed among the tested strains. Strain "LDB/2023/01" demonstrated the highest weight loss of 4.098 grams, showcasing its superior lignin-degrading abilities. The other strains also displayed substantial weight losses, with "LDB/2023/02" showing a weight loss of 3.889 grams, "LDB/2023/03" with 3.209 grams, "LDB/2023/04" with 3.130 grams, and "LDB/2023/05" with 3.107 grams. These findings highlight the varying lignin-degrading capabilities among the different strains, with "LDB/2023/01" being the most efficient lignin degrader among the tested samples. The presence of lignin-degrading enzymes in the strains is evident from the considerable weight loss observed in all samples compared to the blank.

sl No	Sample Name	Initial Weight	Final weight	Difference
1	Blank	239.087 g	236.454 g	2.633 g
2	LDB/2023/01 (*Acinetobacter schindleri*)	237.468 g	233.37 g	4.098 g
3	LDB/2023/02	217.095 g	213.965 g	3.889 g
4	LDB/2023/03	226.367 g	223.158 g	3.209 g
5	LDB/2023/04	242.344 g	239.237 g	3.130 g
6	LDB/2023/05	233.336 g	229.447 g	3.107 g

Table 1 Weight Differences of Incubated Flask Samples

After the 7-day incubation period, the flasks were removed from the shaker, and the contents were subjected to Klason lignin analysis using the acid hydrolysis method. The contents of each flask were centrifuged at 6000 rpm to separate the liquid portion from the solid pellets. The obtained pellets were then subjected to acid hydrolysis, converting the lignin into soluble lignin-derived monomers and other compounds. The resulting hydrolysate was analyzed, and the amount of lignin present in each strain was quantified.

Sample name	Initial weight (g)	Final weight (g)	Difference (g)
LDB/2023/01 (*Acinetobacter schindleri*)	12.525	13.247	0.722
LDB/2023/02	12.597	13.489	0.892
LDB/2023/03	12.431	13.331	0.9
LDB/2023/04	12.383	13.289	0.906

LDB/2023/05	12.546	13.454	0.908

Table 2 results of the Klason lignin analysis for *Acinetobacter schindleri* and other strains

Table 2 presents the results of the Klason lignin analysis for the tested strains and the control sample. The table includes the strain names, their initial weights (weight of the filter paper), final weights (weight after lignin filtration), and the resulting weight differences (weight of the lignin present). Strain " *Acinetobacter schindleri*," which exhibited the highest weight loss during the incubation period, also showed the most significant reduction in lignin content. This finding further supports the strong lignin-degrading potential of strain "*Acinetobacter schindleri* "

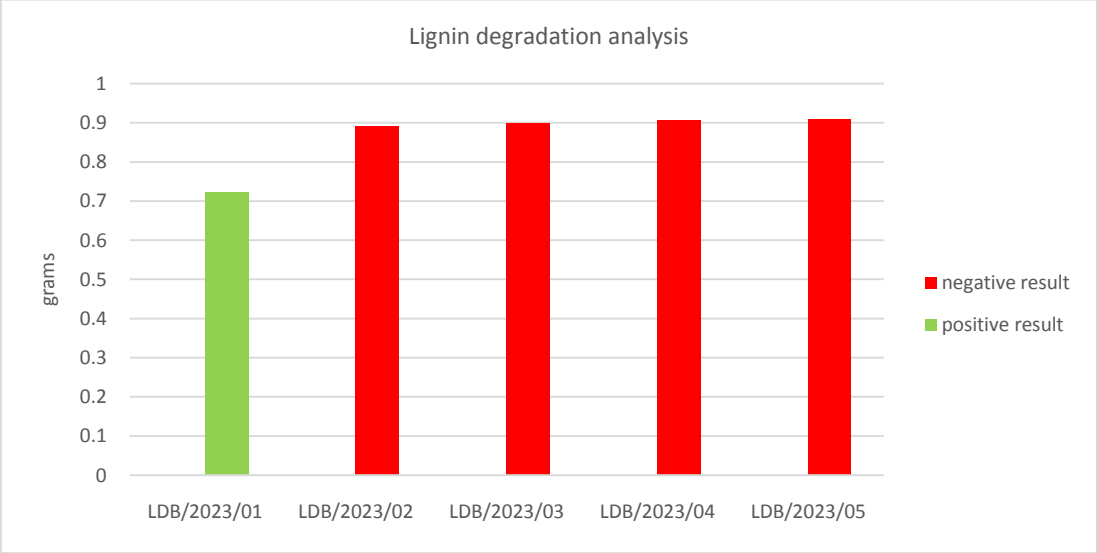

Fig. 3.3 The amount of lignin left after the degradation with each strains.

Fig. 3.3 shows that the strain LDB/2023/01 i.e.*Acinetobacter schindleri*degraded the maximum amount of lignin compared to other bacterial strains. The initial 1gram of lignin was degraded to 0.772gram with the identified bacterial strain. About 0.278gram of lignin was degraded by the *Acinetobacter schindleri.*

IV. Conclusion

The results obtained from the Klason lignin analysis raise intriguing possibilities for further exploration. The superior lignin-degrading capabilities of "LDB/2023/01" make it an exciting candidate for applications in lignocellulosic biomass conversion and environmental remediation. The significant reduction in lignin content suggests that "LDB/2023/01" might harbour unique ligninolytic enzymes or metabolic pathways that enable efficient lignin breakdown.

On the other hand, the other strains, though showing some lignin degradation, did not match the efficiency of "LDB/2023/01." This could be due to variations in the ligninolytic enzyme profiles and metabolic activities among the strains. Further investigation into the enzymatic and metabolic characteristics of each strain will provide a comprehensive understanding of their lignin-degrading potential.

Moreover, extending the incubation period or varying the environmental conditions might enhance lignin degradation in all strains. Lignin degradation is a complex process, and factors such as pH, temperature, and nutrient availability can influence microbial activity. Optimizing these conditions could lead to increased lignin degradation across all strains.

In conclusion, the Klason lignin analysis identified "LDB/2023/01" as the most efficient lignin degrader among the tested strains. Its potential in lignin valorisation and environmental remediation warrants further exploration. However, additional studies are required to decipher the enzymatic and metabolic mechanisms behind its superior lignin-degrading capabilities. Understanding the unique attributes of each strain will pave the way for utilizing lignin-degrading microorganisms effectively in various biotechnological applications and sustainable environmental solutions.

Acknowledgement

The authors of this paper are acknowledged to Gujarat Gas Limited (GGL) of GSPC group for funding the CSR project "Research and development of enriched biogas plant by utilization of agri and bio waste in rural areas".

References

[1] Alves, L., Medeiros, F. H., De Carvalho, A. K. F., Nascimento, R. A., Lopes, A. C. S., & Melo, I. S. (2016). Isolation Of Lignin-Degrading Basidiomycetes Fungi From Amazonian Aquatic Habitats And Their Use In The Decolorization Of Remazol Brilliant Blue R. Water, Air, & Soil Pollution, 227(6), 220.

[2] Boerjan, B. (2003) Chen, Y., Xiong, Y., Zhang, K., Huang, R., & Cao, S. (2020). Identification And Characterization Of Lignin-Degrading Bacteria From Chinese Soil Samples. Frontiers In Microbiology, 11, 1044.

[3] Bugg, T. D. H., Ahmad, M., Hardiman, E. M., &Rahmanpour, R. (2011). Pathways For Degradation Of Lignin In Bacteria And Fungi. Natural Product Reports, 28(12), 1883–1896.

[4] Garcia-Gonzalez, E., Bardaji, L., Lindhoud, S., De Leeuw, M., Abee, T., &Ribo-Arbesú, E. (2013). Isolation Of Endospore-Forming Bacteria With A Relationship To Dairy Industry From Non-Dairy Farm Environments. Plos One, 8(8), E73238.

[5] Gao, M., Kao, T., Zhou, X., Sun, Y., Liu, R., & Qiu, T. (2021). Isolation And Screening Of Lignin-Degrading Bacteria From Rotten Wood For Bioconversion Of Lignocellulosic Biomass. International Journal Of Environmental Research And Public Health, 18(2), 655.

[6] Gupta, A., Verma, J. P., & Yadav, J. (2016). Isolation And Characterization Of Ligninolytic Bacteria For Efficient Kraft Lignin Degradation. Environmental Progress & Sustainable Energy, 35(3), 900-908.

[7] Jones, J. L. (2015). High Throughput Screening Of Seafood For Foodborne Pathogens. In High Throughput Screening For Food Safety Assessment (Pp. 491-505). Woodhead Publishing.

[8] Pandey, V. C., & Kim, K. (2011). Importance Of Rhizosphere Microbial Consortium In Phytoremediation Of Heavy Metals. Reviews Of Environmental Contamination And Toxicology, 213, 273-300.

[9] Ragauskas, A. J., Beckham, G. T., Biddy, M. J., Chandra, R., Chen, F., Davis, M. F., ... & Wyman, C. E. (2014). Lignin Valorization: Improving Lignin Processing In The Biorefinery. Science, 344(6185), 1246843.

[10] Salvachúa, D., Karp, E. M., Nimlos, C. T., Vardon, D. R., Beckham, G. T. (2020). Investigation Of Lignin Degradation In RhodococcusJostii Rha1 By Comprehensive Quantitative Proteomics. Frontiers In Bioengineering And Biotechnology, 8, 541.

[11] Sasikumar, V., Priya, Shankar, C. S., Sekar, D. S. (2014). Isolation And Preliminary Screening Of Lignin Degrading Microbes. Journal Of Academy Of Industrial Research, 3(6), 291-294.

[12] Singh, R. K., Tiwari, M. K., Singh, R., Lee, J. K., & Sharma, S. (2018). Biological Lignin Degradation: Recent Advances, Challenges, And Perspectives. Frontiers In Microbiology, 9, 2207.