Sequential Production of Levan and Xanthan Gum via Integrated Fermentation for Enhanced Carbon Utilization

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Abstract. Microbial exopolysaccharides (EPS) have gained significant attention in recent years due to their diverse applications in food and healthcare sectors. These polymers can exist as either homo- or heteropolysaccharides depending on their monomeric sugar composition. Among these, levan, a fructose-based homopolymer, holds considerable potential in pharmaceutical and food applications. Its biosynthesis by *Bacillus* species using sucrose as the sole carbon source has been extensively studied. Similarly, xanthan gum, a heteropolysaccharide produced by *Xanthomonas campestris*, consists of a β-(1→4)-D-glucose backbone with trisaccharide side chains comprising mannose and glucuronic acid. Xanthan is widely utilized in the food industry as a stabilizer and rheology modifier. *Bacillus* spp. produces levan using a two-step enzymatic process. In the initial step, sucrose is hydrolysed into glucose and fructose. In the second step levan is formed by the polymerization of fructose units. This process leaves a glucose-rich spent medium, which is typically underutilized. The present study proposes a sequential fermentation strategy to valorize the residual glucose and improve overall carbon efficiency. In the first stage, fermentation of 100 g/L of pure sucrose was conducted using *B. subtilis* MTCC 441. Levan was precipitated from the culture broth using isopropyl alcohol (IPA), resulting in a yield of 0.39 g levan/g sucrose. Post-precipitation, the IPA was recovered and the clarified supernatant was concentrated and subjected to sugar profiling via HPLC. The analysis revealed 92.16 g/L of glucose and 10.43 g/L of residual sucrose, indicating significant glucose availability for further processing. To utilize, this residual glucose, the recovered broth was diluted and supplemented with essential micronutrients to formulate a medium containing 20 g/L of glucose for xanthan production. The second-stage fermentation was carried out using *X. campestris* NCIM 2956, which yielded 2.17 g/L of xanthan gum. The structural integrity and identity of the produced EPSs—levan and xanthan—were confirmed using NMR and FT-IR spectroscopy. This study presents a cost-effective and sustainable bioprocessing strategy that integrates levan and xanthan production via sequential fermentation, thereby enhancing carbon conversion efficiency and reducing downstream waste.

1 Introduction

Biopolymers are natural polymers produced by living organisms like plants or microbes that consist of repeating monomeric units covalently bonded to form larger molecules. They have versatile applications, especially in industries like food and medical, owing to their biocompatible, biodegradable and non-toxic nature [1]. Most of the microbial polysaccharides are carbohydrate-based, as these microorganisms transform the carbon and nitrogen into intracellular or extracellular polysaccharides through their unique metabolic pathways [2]. These microbial polysaccharides are usually water-soluble and have a distinct role in the food industries as they are used as stabilisers, emulsifiers, gelling agents, coagulants, etc, to improve the quality and texture of food [1].

Levan is one such fructose-based homopolysaccharide that has unique properties produced mainly using *Bacillus* spp. with sucrose as a primary carbon source. Studies have optimised the production conditions for levan [3]. Another commercially important biopolymer is the xanthan gum, which is produced *by Xanthomonas* sp. with glucose as the carbon source. It is a heteropolysaccharide consisting of repeating units of glucose, mannose, and glucuronic acid [4]. Both levan and xanthan are water-soluble exopolysaccharides with potential applications in the food industry.

During levan fermentation, the sucrose breaks into fructose and glucose, where the fructose molecules polymerise to yield levan by the action of the enzyme levansucrase. A major portion of the glucose produced is not consumed in levan production and hence remains as a byproduct in the fermentation broth. Post-fermentation the

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levan is obtained by precipitation using a suitable anti-solvent like isopropyl alcohol or ethanol. After product recovery, the spent solvent is recovered by distillation, and the spent media is discarded. Since this spent media contains residual fermentable sugars (mainly glucose and traces of sucrose and fructose), it can be converted to a bioproduct to improve the overall economy of the process. The novelty of this study lies in the development of an integrated bioprocess that utilizes residual fermentable sugars, primarily glucose, from levan fermentation spent media for the sequential fermentation of xanthan gum. The approach addresses a critical limitation in conventional levan production, unutilized by product glucose, by enabling its conversion into xanthan gum to improve overall economy of the process. Thus, this study could provide a potential way of producing two commercially important biopolymers (levan and xanthan) and enhancing carbon flux utilisation.

2 Materials and Methods

2.1 Microbial culture conditions

Two bacterial strains, *Bacillus subtilis* MTCC441 (Microbial Type Culture Collection, Chandigarh, India) and *Xanthomonas campestris* (NCIM 2954) (National Collection of Industrial Microorganisms, Pune, India) are used in the study. B. *subtilis* MTCC441 was cultured in Luria Bertani broth for 20 hours at 37°C, and MGYP (malt extract 3g/L, glucose 10g/L, yeast extract 3 g/L) was used for culturing *X. campestris* cells at 30°C for 24 hours.

2.2 Production media formulation and stage-I fermentation

The levan production was formulated according to the optimised protocol as per [3]. Briefly, the medium consists of pure sucrose (100 g/L), yeast extract (2 g/L), ammonium sulphate (3 g/L), potassium dihydrogen phosphate (1 g/L), magnesium sulphate (0.6 g/L), and manganese sulphate (0.2 g/L), with initial pH adjusted to 7 before autoclaving. About 10% (v/v) inoculum was added to 100 mL production media taken in 250 mL conical flask, and the fermentation was carried out at 37°C for 16 hours.

2.3 Levan recovery and residual sugar estimation

After the stage-I fermentation, the cells were centrifuged at 8000 rpm for 10 minutes and kept for drying at 60°C. The supernatant was adjusted to pH 11, and ice-cold isopropyl alcohol (IPA) at a 1:3 (supernatant: IPA) ratio was added. Followed by levan recovery by centrifugation at 8000 rpm for 10 minutes. The precipitated levan was kept for drying at 60°C.

The IPA is recovered from the spent medium using a rotary evaporator and concentrated spent media is obtained. The sugar fraction was analysed using High Performance Liquid Chromatography (HPLC) with a Carbo-sep CHO 782 Pb column equipped with a Refractive index (RI) detector. The sucrose, glucose and fructose remaining after the fermentation were estimated.

2.4 Stage - II fermentation

The xanthan gum fermentation media was formulated by diluting the sample to a glucose concentration of 20 g/L. The media was autoclaved, and 10 % (v/v) of seed culture was inoculated and kept for fermentation at 30°C for 5 days at 200 rpm.

2.5 Xanthan recovery and final sugar estimation

Followed by the fermentation period, the media was autoclaved at 121°C for 10 min. Then the cells were centrifuged at 8000 rpm for 10 minutes. The remaining supernatant was added with 1:3 ice-cold acetone to precipitate the xanthan gum. Finally, the acetone was recovered by distillation, and the residual media were analysed using HPLC.

2.6 Characterisation using NMR and FT-IR

The exopolysaccharides levan and xanthan produced were confirmed using ^{13}C NMR, ^{1}H NMR (JEOL 600 MHz NMR, JNM-ECZ600R/S1), and FT-IR (Perkin Elmer Spectrum 100) analysis. The samples were dissolved in D_2O for NMR analysis.

3 Results

3.1 Levan yield and remaining sugar estimation

In stage-I fermentation, the cell yield was about 3.23 ± 0.18 g/L, and the levan yield of 38.36 ± 0.41 g/L was achieved. The fermentation was started with 10 g of sucrose, following the fermentation levan was precipitated, and the supernatant was rotary evaporated. The amount of residual sugars in the concentrated media was found to be 1.46 g, 3.66 g, and 1.82 g of sucrose, glucose and fructose, respectively. Here, the remaining sucrose corresponds to the unconverted sucrose. The fructose amount is lower due to its dual usage in cell growth and levan production, while the glucose is only used for cell growth and maintenance.

For the stage - II fermentation, the xanthan media was formulated by diluting the supernatant to 20 g/L glucose concentration. Other xanthan production media compounds were supplemented.

3.2 Xanthan yield and final sugar estimation

The stage - II fermentation using *X. campestris* resulted in a biomass yield of 5.135 ± 0.1484 g/L, and xanthan yield of 2.170 ± 0.1838 g/L. The final sugar in the broth was estimated after stage - II fermentation, and the broth contained about 0.319 g of fructose. The residual sucrose was completely consumed by the *X. campestris* for its growth, and hence, sucrose was not present in the

fermented broth. Similarly, glucose concentration was very less (0.033 g) in the broth as the *X. campestris* consumes glucose for its metabolism. The sugar level at each fermentation stage is shown in Figure 1.

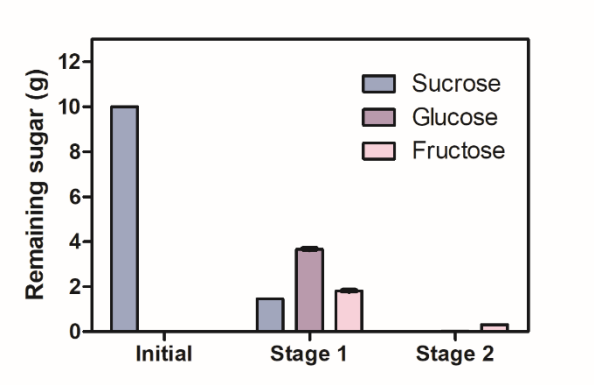
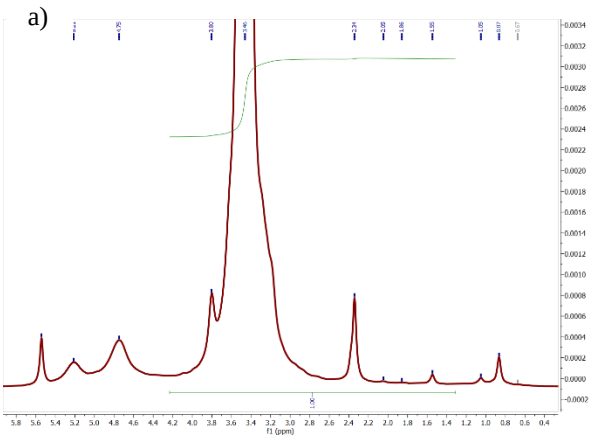


Fig. 1. Sugar remaining in the fermentation broth after each stage of fermentation

3.3 Levan and xanthan confirmation using NMR

Levan is a fructan polysaccharide composed of β (2-6)-linked fructosyl units with an initial α -D-glucopyranose residue, and it displays distinctive NMR characteristics that differentiate it from related fructans such as inulin. In the 1H NMR spectrum, the C6 methylene protons of the fructosyl backbone, indicative of β (2-6) linkages, resonate at 3.66 ppm and 3.49 ppm. Protons at C1 (3.85 ppm), C3 (4.13 ppm), and C4 (4.27 ppm) are attributed to hydrogen-bonding interactions that stabilize the polymer's helical conformation, while the C5 proton of the fructofuranose ring is observed downfield at 4.73 ppm due to deshielding by adjacent glycosidic bonds (Figure 2). The presence of an anomeric proton signal near 5.18 ppm confirms the α-D-glucose unit at the reducing end. In the 13C NMR spectrum, the signals for C2 (104 ppm) and C6 (60.66 ppm) are characteristic of the β (2-6) linkages, while peaks at 80.27 ppm (C5), 76.09 ppm (C3), 75.53 ppm (C4), and 62.09 ppm (C1) correspond to the respective carbons in the fructosyl units [5].



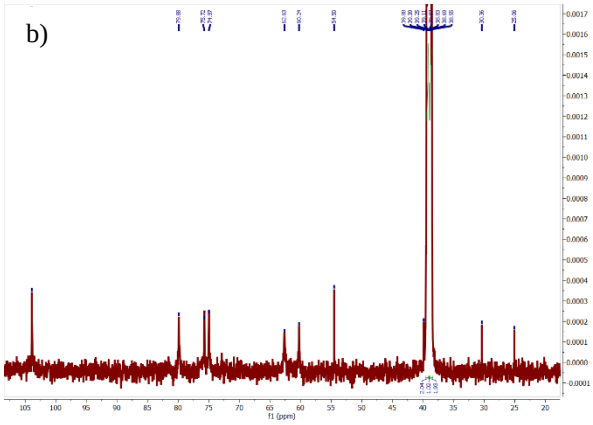


Fig. 2. NMR of levan polymer a). ^{1}H proton, b). ^{13}C carbon.

Xanthan, a cellulose-based polymer, has a side chain consisting of a mannose residue acetylated at C6, linked to galacturonic acid, followed by a terminal mannose bearing a pyruvate group. Proton NMR analysis reveals distinct structural features: the pyruvate methyl group is identified by a resonance at 1.16 ppm, while the acetyl methyl group appears at 1.90 ppm. The anomeric proton of the β -D-glucopyranosyl unit in the cellulose backbone is observed at 4.62 ppm, whereas the α -D-mannopyranosyl anomeric proton resonates at 4.74 ppm. The C5 proton of galacturonic acid, influenced by the carboxylate group, is detected at 4.49 ppm. Broad signals between 3.10–3.58 ppm correspond to hydroxyl protons, consistent with hydrogen bonding in the polysaccharide matrix [6-9]. A separate anomeric proton signal at 5.08 ppm further confirms the mannose residue in the side chain (Figure 3). These assignments align with xanthan's established structure, where acetyl and pyruvate substituents enhance solubility and rheological properties, while anomeric configurations stabilize the helical conformation critical for its functionality.

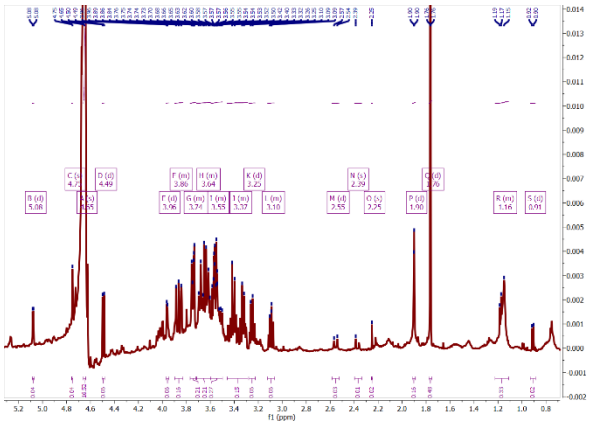


Fig. 3. NMR of xanthan polymer based on ^{1}H proton

3.4 Confirmation using FT-IR analysis

FTIR analysis of levan and xanthan reveals distinct spectral features corresponding to their chemical structures. For levan, characteristic peaks include a strong O–H stretching vibration at 3392 cm^{-1} (hydroxyl group), a C–C stretch at 2929 cm^{-1} indicative of the furanose ring, a C–O stretch at 1346 cm^{-1} within hydroxyl groups, and a prominent ether linkage (C–O–C) at 1114 cm^{-1}. In contrast, xanthan shows a broad O–H stretch at 3450 cm^{-1} (hydroxyl groups), C–C and C–H stretching bands at 2883 cm^{-1} and 2817 cm^{-1} (both related to the furanose ring), and notable carbonyl absorptions at 1765 cm^{-1} (carboxylic acid) and 1633 cm^{-1} (acetyl group). Additional peaks include a C–O stretch at 1415 cm^{-1} (hydroxyl group) and an ether linkage (C–O–C) at 1117 cm^{-1}, further confirming the polysaccharide backbone and functional diversity of xanthan (Figure 4).

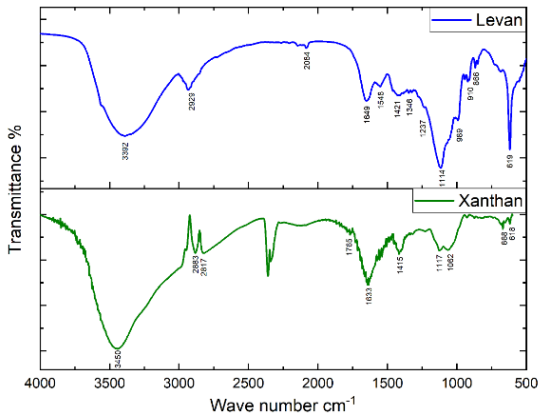


Fig. 4. FT-IR analysis of levan and xanthan

4 Discussion

Studies have demonstrated the co-production of polyhydroxyalkanoates (PHA) along with biohydrogen [10]. In a similar attempt by [11], co-production of PHB and violacein was achieved using *Iodobacter* sp PCH194 isolated from the Himalaya. Even the co-production of PHA and an exopolysaccharide was achieved [12] from pine hydrolysate as an alternative carbon source. In an attempt by [13], they have achieved co-production of P3HB (53 %) and gluconic acid (133 g/L) using *Halomonas elongate*. In a similar study by [14], they have co-produced PHA along with ethanol, where they have investigated on the impact of ethanol on carbon flux of the PHA production. All these studies have produced PHA or PHB polymer, which are usually obtained along with the cell biomass, and the supernatant contains the exopolysaccharides [14]. In an attempt by [16], they have achieved sequential production of PHA along with levan [17]. has previously reported sequential production of levan with poly-ε-lysine through microbial fermentation using sucrose as substrate. Therefore, we have tried to produce levan along with xanthan gum through microbial fermentation.

Here, in our presented study, we aimed to produce two biopolymers (levan and xanthan), both of which are exopolysaccharides secreted in the fermentation broth. Initially, the fermentation was started with sucrose (10 g) as a carbon source for the levan production. We were able to get around 38.36 ± 0.41 g/L of levan after the first fermentation. Following this, the media is left with 1.46 g of sucrose, 3.66 g of glucose and 1.82 g of fructose. For xanthan production, the left-out media was diluted five times to attain a 20 g/L (1.98 g) of glucose concentration, while the fructose of 0.98 g and sucrose of 0.79 g remained in the media. Upon the second fermentation, xanthan was produced at 2.17 ± 0.184 g/L. The final media remaining was left with 0.0328 g of glucose, 0.319 g of fructose and zero sucrose. From the results, we can infer that the *X. campestris* was able to consume sucrose and glucose to the maximum extent, along with fructose in moderation. Therefore, for further application of this study may include the strategy to utilize hydrolysate sugars obtained from biorefining process of lignocellulose biomass [18] for productions of levan and xanthan. This target will help to support the sustainable development goals, especially SDG12 and circular economy to reduce consumption of natural resources [19].

5 Conclusion

The production of levan and xanthan gum was achieved using a sequential fermentation method. From the results, we can observe that the spent media stream after the fermentation of levan can be efficiently used for the production of xanthan gum, in sequential fermentation. It can also be observed that the remaining sucrose and some amounts of fructose are also being utilised by the *X. campestris*, as it has the ability to consume both mono and disaccharides. From the study, it can be seen that one of its strengths lies in the efficient utilization of resources. This is a notable advantage, as research on the application of fructose is less common compared to other types of sugars. Therefore, this study has the potential to be further developed to add value in industrial applications. This integrated strategy helps in maximising the resource by improving the overall economic feasibility.

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References

1. J. Baranwal, B. Brajesh, F. Antonella, L. D. Giovanna, K. Amit, Biopolymer: A Sustainable Material for Food and Medical Applications, Polymers. **14**, 983 (2022). https://doi.org/10.3390/polym14050983
2. M. Sharma, T. Nihed, K. Imen, R. Tarek, Microbial Biopolymers: From Production to Environmental Applications—a Review, Applied Sciences. **14**, 5081 (2024). https://doi.org/10.3390/app14125081
3. Chidambaram, J. C. A. Sailaja, V. Bhuvaneshwari, K. S. Kartik, M. P. Krishna, R. S. Saravanan, V. Ponnusami, Studies on Solvent Precipitation of Levan Synthesized Using Bacillus Subtilis MTCC

441, Heliyon **5**, e02414 (2019). https://doi.org/10.1016/j.heliyon.2019.e02414

4. H. Li, X. Ronge, W. Zongji, L. Guantian, Advancements in Xanthan Gum-Based Film and Coating for Food Packaging, Carbohydrate Polymers. **356**, 123409 (2025). https://doi.org/10.1016/j.carbpol.2025.123409
5. B. Veerapandian, S. R. Shanmugam, S. Varadhan, K. K. Sarwareddy, K. P. Mani, V. Ponnusami, Levan Production from Sucrose Using Chicken Feather Peptone as a Low Cost Supplemental Nutrient Source, Carbohydrate Polymers. **227,** (2020). https://doi.org/10.1016/j.carbpol.2019.115361
6. S. Faria, L. O. P. Carmen, A. L. M. Sérgio, G. H. T. Manuel, M. R. Miriam, P.F. Francisca, L. C. Vicelma, Characterization of Xanthan Gum Produced from Sugar Cane Broth, Carbohydrate Polymers. **86**, 469–76 (2011). https://doi.org/10.1016/j.carbpol.2011.04.063
7. J. Kang, Y. Hongxia, L. Xinxue, H. Chao, L. Qin, C. Liting, Z. Jixiang, L. Yan, W. Shujun, G. Qingbin, Structural, Rheological and Functional Properties of Ultrasonic Treated Xanthan Gums, International Journal of Biological Macromolecules. **246**, 125650 (2023). https://doi.org/10.1016/j.ijbiomac.2023.125650
8. M. Rinaudo, M. Milas, F. Lambert, M. Vincendon, Proton and Carbon-13 NMR Investigation of Xanthan Gu. ACS Publications. (2002) https://doi.org/10.1021/ma00239a018
9. M. Takemasa, N. Katsuyoshi, Solution Structure of Molecular Associations Investigated Using NMR for Polysaccharides: Xanthan/Galactomannan Mixtures. *Journal of Physical Chemistry B.* **120**, 3027–37 (2016). https://doi.org/10.1021/acs.jpcb.5b11665
10. D. Thulasidharan, A. Arumugam, B. U. Kiran, Research and Economic Perspectives on an Integrated Biorefinery Approach for the Simultaneous Production of Polyhydroxyalkanoates and Biohydrogen. *International Journal of Biological Macromolecules.* **193**, 1937–51 (2021). https://doi.org/10.1016/j.ijbiomac.2021.11.025
11. V. Kumar, D. Sanyukta, K. Subhash, K. Sanjay, S. Dharam, Bioprocess for Co-Production of Polyhydroxybutyrate and Violacein Using Himalayan Bacterium *Iodobacter* sp. PCH194. Bioresource Technology. **319**, 124235 (2021). https://doi.org/10.1016/j.biortech.2020.124235
12. S. K. Bhatia, G. Ranjit, K. Byungchan, K. Suhyun, C. Do-Hyun, J. Heeju, K. Yun-Gon, K. Jae-Seok, Y. Yung-Hun, Coproduction of Exopolysaccharide and Polyhydroxyalkanoates from *Sphingobium Yanoikuyae* BBL01 Using Biochar Pretreated Plant Biomass Hydrolysate, Bioresource Technology. **361**, 127753 (2022). https://doi.org/10.1016/j.biortech.2022.127753
13. T. Leandro, M. Conceição Oliveira, M. Manuela R. da Fonseca, M. Teresa Cesário, Co-Production of Poly(3-Hydroxybutyrate) and Gluconic Acid from Glucose by Halomonas Elongata, Bioengineering. **10**, 643 (2023). https://doi.org/10.3390/bioengineering10060643
14. C. Rangel, D. L. Nídia, A. M. R. Maria, C. Gilda, Dynamics in the Profile of Biopolymers Produced by Mixed Microbial Cultures from Ethanol-Rich Feedstocks, Journal of Environmental Chemical Engineering. **12**, 112609 (2024). https://doi.org/10.1016/j.jece.2024.112609
15. de Siqueira, C. Edmilson, A. A. Aline, E. C. S. Páblo, P. S. B. Maria, M. H. Laureen, Polyhydroxyalkanoates and Exopolysaccharides: An Alternative for Valuation of the Co-Production of Microbial Biopolymers, Biotechnology Progress. **40**, e3412 (2024). https://doi.org/10.1002/btpr.3412
16. E. C. Siqueira, M. H. Laureen, Sequential Production of Two Biopolymers-Polyhydroxyalkanoate and Levan by Microbial Fermentation, Research, Society and Development. **12**, e25212340745–e25212340745 (2023). https://doi.org/10.33448/rsd-v12i3.40745
17. I. L. Shih, W. Tsaur-Chin, C. Shou-Zoo, L. Gen-Der, Sequential Production of Two Biopolymers-Levan and Poly-ε-Lysine by Microbial Fermentation, Bioresource Technology. **102**, 3966–69 (2011). https://doi.org/10.1016/j.biortech.2010.11.066
18. S. Areeya, E. J. Panakkal, P. Kunmanee, A. Tawai, S. Amornraksa, M. Sriariyanun, A. Kaoloun, N. Hartini, Y. S. Chen, M. Kchoau, S. Dasari, M. P. Gundupalli, A review of sugarcane biorefinery: From waste to value-added products, Applied Science and Engineering Progress. **17**, 7402 (2024). https://doi.org/10.14416/j.asep.2024.06.004
19. R. J. P. Latiza, R. V Rubi, Circular economy integration in 1G+2G sugarcane bioethanol production: application of carbon capture, utilization, closed-loop systems and waste valorization for sustainability, Applied Science and Engineering Progress. **18**, 7448 (2025). https://doi.org/10.14416/j.asep.2024.07.005