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Biotransformation of Sugar-Containing Wastewater into α-1,3-Glucan Based Bioplastic

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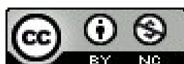
Abstract

With the advent of synthetic biology, wastewater is turning into a resource, especially from sugar industry wastewater, which is rich in organic matter. Efficient transformation and purification of synthetic products is the bottleneck for the recycling and reuse of wastewater. To address this issue, here we extracted a gene expressing glucosyltransferase from *Streptococcus salivarius* and engineered it into the bacterium *Bacillus subtilis* for the secretion of glucosyltransferase to transform sucrose in the wastewater into α-1,3-glucan which is prone to precipitate, thus facilitating purification. The α-1,3-glucan polysaccharide harvested from wastewater was then turned into bioplastic film through oxidative double crosslinking, which showed a synergistic effect on the mechanical strength of bioplastic film. Compared to common bioplastic and chemically crosslinked bioplastic, the stress-strain curves, tensile strength and Young's modulus were significantly higher in double crosslinked bioplastics. In addition, the thermal decomposition rate of the double cross-linked bioplastics was found lower than the other two bioplastic materials. As a proof of concept, this strategy paved the way for water recycling and eco-friendly material production.



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Introduction

With the ongoing advancement of industrialization and the improvement in people's quality of life, sugar has become an essential part of daily life [1]. In China's sugar crop industry, the primary crops include sugarcane, sugar beet, and sugar sorghum. In the industrialized production of sugar, the treatment of large volumes of sugar-containing wastewater is a critical issue. Finding economical and efficient ways to utilize this wastewater is a significant challenge. The main by-products of sugar-containing wastewater, apart from sugar, include molasses, bagasse, and filter cake [2]. Through the persistent efforts of numerous scientific researchers, economical and efficient methods for utilizing these by-products have been developed. For example, Hawaz et al. [3] examined the broad application of molasses in biofuel production and optimized its fermentation process to produce bioethanol through continuous experiments. Additionally, Fuess and colleagues investigated the feasibility of bio-hydrogen production from molasses, while also producing free amino acids, antioxidants, and other valuable products [4-6].

Since bagasse was primarily burned in piles, improving air supply is necessary to achieve optimal combustion efficiency. In response to this, Cundy et al. [7] designed an advanced incinerator to ensure complete combustion, thereby reducing the moisture content of bagasse and minimizing heated loss. Filter cake, on the other hand, was widely employed as an organic fertilizer to enhance agricultural soil quality, most commonly as a replacement for potassium and phosphorus [8]. Thus, wastewater could be considered a misallocated resource. Additionally, the high sucrose content in this wastewater has drawn our attention. This raises several questions: Could sucrose be biotransformed and could this process achieve economic viability while remaining environmentally benign? Polysaccharides from various sources are regarded as some of the most crucial resources for the development of sustainable materials, both now and in the future [9]. Currently, starch and cellulose are widely utilized in engineering applications. However, as renewable resources, engineered polysaccharides produced through enzymatic polymerization of different carbon sources have garnered significant attention and interest [10]. In line with the concept of wastewater recycling, this experiment aimed to convert sucrose in the collected sugar wastewater into α-1,3-polyglucose and fructose using glycosyltransferase, thereby obtaining α-1,3-glucan [11].

As a biomaterial, dextran has garnered significant attention due to its unique properties, including biocompatibility, non-toxicity, biodegradability and water solubility. It possessed considerable use value in diverse fields [12-16]. As a specific type of glucan, α-1,3-glucan is an α-1,3-linked glucose homopolymer and a water-insoluble polymer [17]. Currently, there is a significant commercial demand for pure α-1,3-glucan in the market, which has led to considerable interest in its applications [18-24]. To fully harness the potential of α-1,3-glucan, it has been modified both physically and chemically. Meanwhile, the environmental impact of disposable non-degradable plastics has garnered global attention, prompting countries worldwide to implement relevant policies for regulation [25]. Thereupon, our research group has proposed the utilization of α-1, 3-glucan derived from sucrose in sugar-containing wastewater as a raw material for the production of bioplastics, aiming to mitigate the environmental pollution caused by plastic products derived from fossil resources and simultaneously achieved a win-win strategy of converting waste into valuable resources.

Materials and Methods

Experimental materials

The sugar wastewater from Nanjing Sweet Juice Garden Sugar Factory was used as raw material. Liquid Luria-Bertani (LB) medium (5g of yeast extract, 10g of tryptone and 10g of sodium chloride) pH 7.0 was used for routine microbial growth. For the fermentation medium, five different solutions and media were prepared as follows: A: 1% peptone and 0.5% yeast extract; B: 1.25M disodium hydrogen phosphate, 1.25M potassium dihydrogen phosphate, 2.5M ammonium chloride and 0.25M sodium sulfate; C: 25% glycerol + 2.5% glucose; D: 1M magnesium sulfate and E: 8.13 g/L $FeCl_3$, 2.2 g/L $CaCl_2$, 1.98 g/L $MnCl_2·4H_2O$, 2.88 g/L $ZnSO_4·7H_2O$, 0.5 g/L $CoCl_2·6H_2O$, 0.48 g/L $Na_2MoO_4·2H_2O$, 0.12 g/L H_3BO_3 dissolved in 60mM HCl. These different solutions and media were mixed as 48 ml A, 1 ml B, 1 ml C, 100 µl D and 50 µl E. A, B, C and D were sterilized at high temperature separately, and E was filtered for sterilization. Other solutions were 0.1 M HCl, 0.5 M NaOH and 0.1 M $CaCl_2$. The AGU solution (glucose unit: $C_6H_{10}O_5$): ECH (epichlorohydrin): α-1,3-glucan = 1: 1: 1 was prepared. Because 10 g of α-1,3-glucan was added, the molar mass of glucose unit was 162 g/mol, and

the relative molecular mass of epichlorohydrin was 92.524 g/mol, 0.0617 mol of glucose unit and 0.0661 mol of epichlorohydrin were needed.

Gene synthesis of improved GtfJ enzyme

To enhance the production efficiency of α-1,3-glucan synthesized from sucrose, GtfJ enzyme was optimized. The BamHI-GtfJ-NotI gene expressing glucosyltransferase was extracted from *Streptococcus salivarius* by enzyme digestion. Then, it was ligated with pET28a and digested by the same enzyme to form plasmid pET28a-GtfJ. BamHI-GtfJ-NotI was digested from pET28a-GtfJ, and was linked by Gibson at XbaI site of pBA300 to form pBA300-GtfJ. The pBA300-GtfJ plasmid was transfected into *Bacillus subtilis* WB800N by electroporation to obtain WB800N/pBA300-GtfJ.

Induced enzyme production

We took 15 ml of prepared LB medium, put it into a centrifuge tube, added 100 µl of kanamycin as an antibiotic, added the obtained *Bacillus subtilis* WB800N, put it in a shaker, cultured at 37℃ and activated it for 16 h. Took 1 ml of activated culture solution, put it into a centrifuge tube, used tap water as a reference, and measured its OD value with an ultraviolet spectrophotometer at 600 nm. After the test, pour the remaining liquid into the centrifuge tube (there is still a small amount left at the bottom of the centrifuge tube), add water to dilute it to 1 ml, put it into a centrifuge for 1 min at 12000 rpm, poured the supernatant, dropped the remaining turbid liquid on the glass slide, dyed it by Gram's semi-staining method, and conducted microscopic examination under an oil mirror. The microscopic examination should observe that there is no obvious hetero-bacterial infection. Next, injected 1:100 of the bacterial solution (the volume ratio of bacterial liquid to fermentation medium) into the fermentation medium, added 700 µl of 50 mg/mL kanamycin, put it in a shaker, cultured at 37℃, and mixed well until the OD value was between 0.6 and 0.8. The cultured bacteria were induced by adding 600 µl IPTG (isopropyl -β-D-thiogalactoside) inducer and then cultured in a shaker at 16℃, 18 h and 200 rpm. The culture was transferred into a centrifuge bottle for centrifugation at 10000 rpm for 20 min. Dumped the supernatant, added the lysate, suspended the bacterial weight in the lysate with a stirring rod, centrifuged again and discarded the supernatant. Prepared 500 mg/ml lysozyme/lysate mixed solution, added 120 ml mixed solution into a centrifuge bottle, suspended the weight of bacteria in the mixed solution with a stirring rod, poured it into a 250 ml conical flask, put it into a shaker and cultivated at 28℃ for 1.5 h. Later, 20 ml of the cultured suspension was added into a centrifuge tube and performed ultrasonic crushing under the condition of ice-water bath, with 50% power and 5 s intervals for 40 min. The product after ultrasonic crushing was subpackaged and centrifuged in an ice-water bath at 12000 rpm at 4℃ for 20 min. The supernatant was collected as an improved enzyme solution and refrigerated at -55℃ for later use.

Enzymatic synthesis

We collected sugarcane wastewater, filtered by vacuum, evaporated and concentrated, centrifuged, dried and separated it into sucrose crystals. Weighed 5.78 g citric acid monohydrate and 21.32 g trisodium citrate, completely dissolved them, then adjusted the pH to 5.5, and adjusted the volume to 1 L. Using citric acid buffer as solvent, 200 ml of sucrose solution (342.3 g/L) was prepared in 500 ml conical flask, followed by the addition of 12 ml of enzyme, and it was allowed to stand for 24 h at 30℃. When the enzymatic reaction was finished, the sample was transferred to a centrifuge bottle and centrifuged for 30 min at 10000 rpm. The supernatant was poured off, added sterile water to resuspend the obtained glucan, centrifuged again, repeated the operation three times, and after the last centrifugation, added pure water to make the weight of α-1,3-glucan equal to that of the initial enzymatic reaction system, and stored it in a refrigerator at -4℃ for later use.

Double crosslinking product plastic

Put a part of the obtained α-1,3- glucan into a centrifuge bottle, centrifuged at 10000 rpm for 30 min, dump the supernatant, and store the obtained pure α-1,3-glucan in a refrigerator at -4℃ for later use. Took 0.16 g 2,2,6,6-tetramethylpiperidinyl-1-oxide (TEMPO), 1 g of NaBr and 62 g of 6% NaClO solution, dissolved it in 1 L of pure water, adjusted the pH of the solution to 10 with 0.1 M HCl, added 10 g of α-1,3-glucan to the solution while stirring to trigger TEMPO oxidation, and added 0.5 M NaOH solution to keep the pH of the oxidation solution at 10 until no NaOH consumption is observed. Put the obtained TEMPO-oxide into a centrifuge bottle, centrifuged for 10 min at 4000 rpm, poured the supernatant into a beaker, measured the pH value of the supernatant, added pure water to the centrifuge bottle, suspended the TEMPO-oxide with a stirring rod, centrifuged again, repeated the operation several times until the pH of the supernatant becomes 7, added pure water to the centrifuge bottle, suspended

the TEMPO-oxide with a stirring rod, then poured the oxide solution into a large beaker and put it in. The obtained oxide solution was subpackaged into 50 ml centrifugal tubes, put into an ultrasonic crusher under the condition of the ice bath, and crushed for 1 h with 50% power and 5s intervals. Put the product into a centrifuge bottle, centrifuged at 4000 rpm for 10 min to ensure high dispersion uniformity, poured the supernatant, and adjusted it to 1.0% by weight with pure water (the mass of oxide accounts for 1.0% of the total mass). Later, the vacuum filtration device was assembled and 10 ml of TEMPO-α-1,3-glucan dispersion was spread on a PTFE membrane (bore diameter 0.1 μm, diameter 47 mm), which was vacuum filtered to form a wet matrix, and then dried at 40℃ and 90% relative humidity for 12 h, so that a normal bioplastic film could be obtained. Later, 5.7114 g of Epichlorohydrin (ECH) was added to the TEMPO-α-1,3-glucan system, and the mixture was stirred for 1 h and the chemical crosslinking reaction was carried out for 12 h at room temperature. A total of 10 mL of TEMPO-α-1,3-glucan dispersion was spread on the PTFE filter membrane and the wet matrix was formed by vacuum filtration. Immersed the wet substrate in 0.1 M $CaCl_2$ solution for 8 h physical crosslinking, put the obtained double crosslinked product into a centrifuge bottle, centrifuged at 4000 rpm for 10 min, poured the supernatant into a beaker, measured the conductivity value of the supernatant, added pure water into the centrifuge bottle, suspended the double crosslinked body with a stirring rod, centrifuged again. This operation was repeated several times until the conductivity value of the supernatant was constant and dried for 12 h at 40℃ and 90% relative humidity.

Results and Discussion

Detection of modified glucosyltransferase

Sucrose was converted into α-1,3-glucan under the action of glucosyltransferase, which could be stored at -4℃. It could be seen that α-1,3-glucan is a potential low-cost polymer. In this experiment, the glucosyltransferase gene was modified in *Bacillus subtilis*. The genome of *Streptococcus sialus* ATCC 25975 was extracted using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen, USA). We used primers F1 and R1 to amplify the GtfJ gene (F1: GAAGGAGAAAGGTACATGCACATCACCATC ACCATGAGAATAAAATAC; R1：ATGGTACCT TTCTCCTCTTTAGTTCAGA). Then we ligated the amplified product with pET28a plasmid digested by BamHI and NotI and constructed the recombinant plasmid of pET28a-GtfJ. Using it as a template, the PBA300-GTFJ recombinant vector was constructed by amplifying the GtfJ gene fragment with primers F2 and R2 (F2: GCCGATTACAAAAACATCAGCCCA CCATCACCATCACCATGAG; R2: GCCCGCTCA TTAGGCGGGCTGCTTAGTTCAGAACGCGCGG CGGATAC), followed by ligation with the XbaI-digested pBA300 vector using Gibson Assembly. The recombinant vector PBA300-GTFJ was then transformed into *Bacillus subtilis* WB800N, yielding the modified strain *Bacillus subtilis* WB800N/pBA300-GtfJ, which expressed the modified glucosyltransferase named GtfJ-6. The target genes were subsequently verified by gel electrophoresis (Fig. 1). It could be seen that the length of the gene separated by PCR amplification and agarose gel electrophoresis was 1500bp, which proved that the gene was the modified glucosyltransferase gene we constructed.

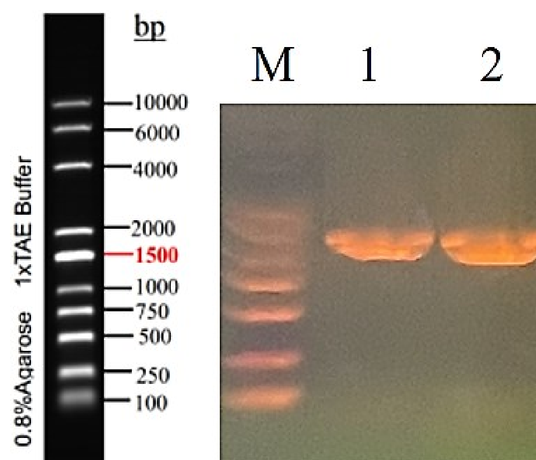

Fig. 1 Gel electrophoresis to verify the glucosyltransferase gene transformed into *Bacillus subtilis*.

Growth curve of modified *Bacillus subtilis*

The obtained modified *Bacillus subtilis* was cultured to prepare for the subsequent enzymatic reaction to produce α-1,3- glucan, and its growth curve was determined (Fig. 2). The abscissa was the growth time of modified *Bacillus subtilis,* and the ordinate was the absorbance of modified *Bacillus subtilis* at 600 nm. The greater the absorbance, the greater the concentration of modified *Bacillus subtilis*, and the better its growth. It was evident that the 0-2 hours period represents a lag phase for the modified *Bacillus subtilis*, indicating an initial adaptation to the new environment following inoculation into the medium. This adaptation was reflected in the flat and stable curve on the line chart, as bacterial

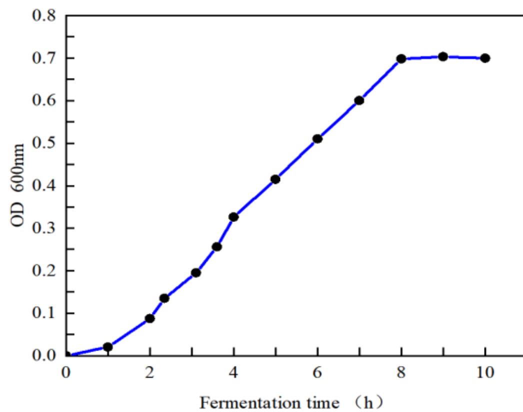

Fig. 2 Growth curve of modified *Bacillus subtilis*.

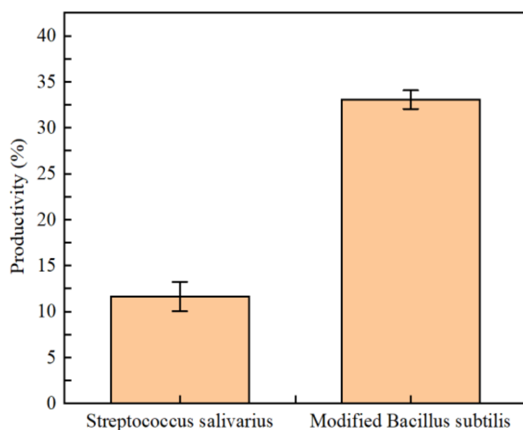

Fig. 3 Yield of α-1,3-glucan produced by different enzyme-producing bacteria.

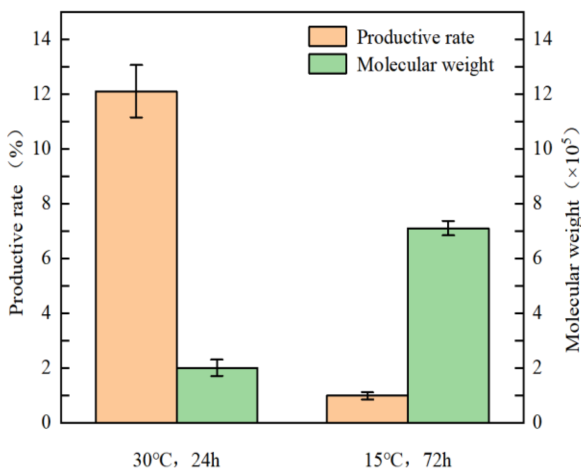

Fig. 4 Yield and molecular weight of α-1,3- glucan produced at different temperatures and enzymatic reaction times.

multiplication was minimal during this time. Despite limited growth, *Bacillus subtilis* undergoes significant intracellular changes, including increased cell volume, active metabolism, synthesis of enzymes, energy reserves, and intermediate metabolites necessary for subsequent division and proliferation. From 2 to 8 hours, the bacteria entered a logarithmic growth phase, characterized by a sharp increase in viable cell count on the growth curve. During this phase, the morphology, staining characteristics, and biological activity of *Bacillus subtilis* were at their most typical, making them highly sensitive to external environmental factors. Between 8 and 10 hours, the growth of *Bacillus subtilis* transitioned into a stationary phase. While the total number of living bacteria remained relatively constant, there was a marked change in population vitality. Factors such as nutrient depletion, accumulation of toxic byproducts and pH reduction contributed to a gradual decrease in reproduction rate and an increase in relative death rate. At this stage, the rates of proliferation and mortality began to balance. Additionally, changes in morphology, staining, and biological activity may occur, along with the production of specific metabolites.

Comparison of glycosyltransferase production efficiency before and after modification

In order to more directly feel the advantages of the modified glucosyltransferase gene, we used *Streptococcus salivarius* and the modified glucosyltransferase in *Bacillus subtilis* to perform enzymatic reactions to produce α-1, 3-glucan, respectively. After culturing at 30℃ for 24 h, the reaction efficiency of the two glucosyltransferases was characterized by the yield of α-1, 3-glucan obtained (Fig. 3). It could be seen that under the same reaction conditions, the amount of α-1, 3-glucan produced by GTFJ-6 is much higher than that of GtfJ gene. Specifically, during the reaction process at 30℃ and 24 h, glucosyltransferase in *Streptococcus salivary* was used for enzymatic reaction, and the yield of α-1, 3-glucan obtained was 11.7% on average. The average yield of α-1, 3-glucan was 33.1% when the glucosyltransferase in the modified *Bacillus subtilis* was used for enzymatic reaction.

Comparison of yield and molecular weight at different temperatures and reaction time

As a polysaccharide, α-1,3- glucan had different molecular weights and yields at different temperatures and different reaction times, which had an important impact on the subsequent application of α-1,3- glucan. Therefore, we cultured at 30℃ for 24 hours and at 15℃ for 72 hours, respectively. The molecular weight and yield of the obtained product were measured (Fig. 4). Interestingly, at lower

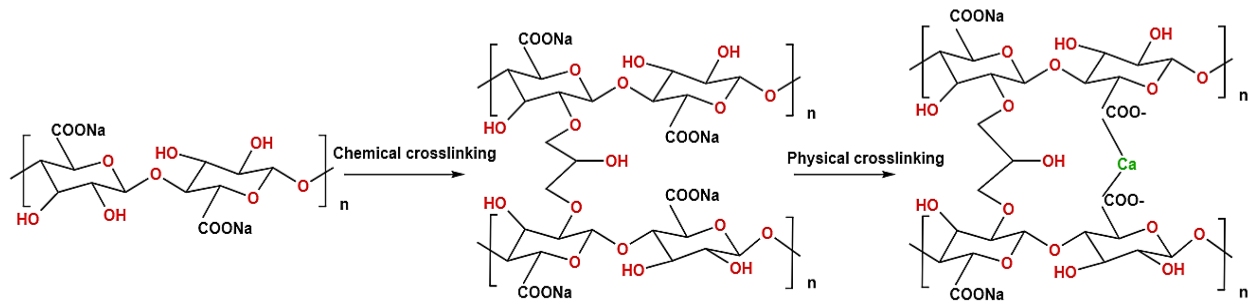

Fig. 5 Physical-chemical double crosslinking process of oxidation complex.

temperatures, the amount of active chain generated by chain initiation was lower, which led to a larger molecular weight and lower yield of α-1,3- glucan.

Cross-linking to produce bioplastics

During the reaction, TEMPO-α-1,3- glucan oxidation complex mainly relied on hydroxyl and carboxylate in the structure for continuous physical and chemical cross-linking reaction to prepare double cross-linked bioplastic films (Fig. 5). TEMPO-α-1,3- glucan oxidation complex could be obtained by mechanical homogenization of α-1,3-glucan under TEMPO-mediated oxidation. ECH was used as a specific chemical crosslinking agent, which was mixed with the oxidation complex to form a domain for chemical crosslinking. After the chemical crosslinking reaction, it was immersed in a calcium chloride solution with the aid of vacuum filtration to induce physical crosslinking. The purpose was to replace sodium ions in the oxidation complex with calcium ions, which could enhance the mechanical properties of the obtained bioplastic film.

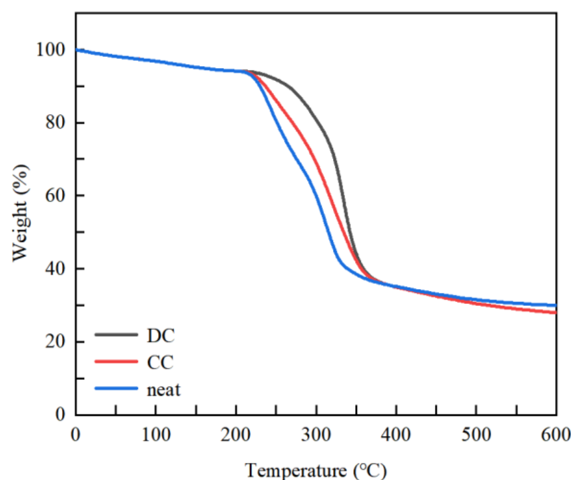

Fig. 6. Thermal stability under different cross-linking methods. Common bioplastics (neat), chemically crosslinked bioplastics (CC) and double crosslinked bioplastics (DC).

Comparison of physical properties of bioplastics with three cross-linking methods

Next, the thermodynamic stability of plasticized common bioplastics (neat), plasticized chemically crosslinked bioplastics (CC) and plasticized double crosslinked bioplastics (DC) was evaluated by thermogravimetric analysis (Fig. 6), in order to explore whether double crosslinking would enhance the thermal stability of bioplastics. It can be found that the temperature rise range was 0-600°C, and the whole curve should be divided into three stages: in the process of 0-220°C, the weight loss curve was relatively stable, mainly due to the escape of adsorbed water. It was generally believed that crystallization water and combined water would be lost at about 100-120°C, and if the material contains low-boiling solvent residues, it would also be volatilized in this process. In the process of 220-370°C, the weight decreased sharply and the slope increased gradually, which indicated that the sample began to degrade thermally, and most organic side chains began to decompose further at 250-300°C, and the crystal structure also began to degrade by oxidation. In the process of 370-600°C, the quality tended to be stable gradually, which showed that the thermal decomposition of the material was basically completed. Similarly, in order to study the influence of the double crosslinking strategy on the mechanical properties of bioplastics, we measured the stress-strain curves, tensile strength and Young's modulus of the three materials respectively (Fig. 7A-C). The results showed that the tensile strength and Young's modulus (180MPa, 16GPa) of pure TEMPO-α-1,3-glucan oxidation complex were significantly lower than those of double-crosslinked bioplastic films (270MPa, 23GPa). It can be confirmed that the double cross-linking reaction has a synergistic effect on the mechanical properties of bioplastic films. According to experience, the mechanical properties of double cross-linked bioplastic films were higher

than the tensile strength and Young's modulus of PET films (247.9MPa, 4.3GPa), as well as those of PE plastic bags (36.4MPa, 3.3GPa).

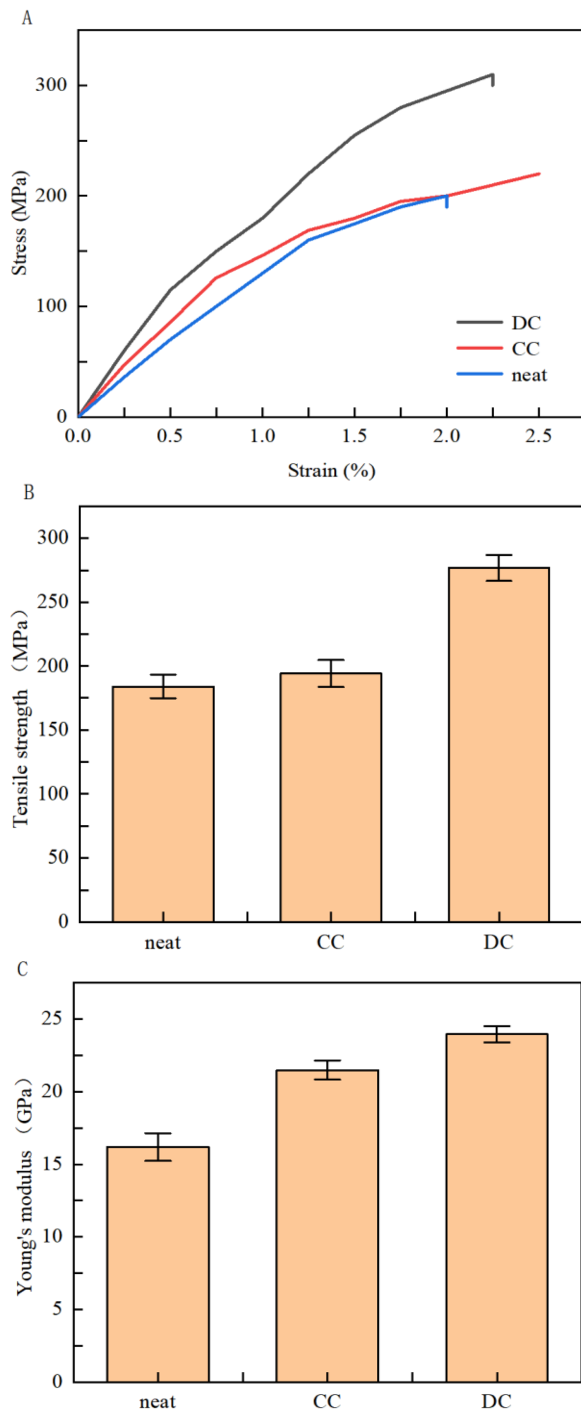

Fig. 7 Strain-stress changes under different cross-linking methods (A). Tensile strength under different cross-linking methods (B) and Young's modulus under different crosslinking modes (C). Common bioplastics (neat), chemically crosslinked bioplastics (CC) and double crosslinked bioplastics (DC).

Overall, we would choose to prepare α-1,3- glucan by culturing modified *Bacillus subtilis* at 30℃ for 24 hours at the initial stage of stability. At the initial stage of stabilization, *Bacillus subtilis* was in a state of stable growth, with certain biological activity, which could complete the expression of protein in cells. At the same time, it would not lead to cell depletion and toxin enrichment due to excessive energy consumption. At the same time, the GtfJ-6 gene expressed by modified *Bacillus subtilis* had better reaction efficiency and more α-1,3-glucan with lower molecular weight could be obtained by culturing at 30℃ for 24 hours, which could ensure the uniform dispersion of the material in the subsequent reaction process. In addition, the physical properties of the three materials were measured, and it could be found that the thermal stability curves of the three materials are similar, but the thermal decomposition rate of the double cross-linked bioplastics was lower than that of the other two materials. It could be seen that the double cross-linking method could enhance the thermal stability of organisms to some extent. Similarly, the tensile strength and Young's modulus of double cross-linked materials were significantly higher than those of the other two materials, which proved that physics and chemistry have synergistic effects on the mechanical properties of bioplastics. According to the formula of Young's modulus:

$$E = \frac{\sigma}{\varepsilon}$$

Among this equation, E stood for Young's modulus, σ stood for normal stress and ε stood for normal strain. Young's modulus was large, indicating that the deformation of the material was small when it was compressed or stretched. In general, the double cross-linking method could make the material stronger, but the material processed in this way lacks certain plasticity and we needed to adjust according to the specific use. So, industrialization still had a long way to go.

Conclusions

In summary, this paper put forward a green and efficient method to construct double cross-linked bioplastic films. Sucrose in sugar-containing wastewater was used as raw material and glucosyltransferase GtfJ-6 was added to generate the intermediate α-1,3-glucan, and then ECH and calcium chloride were used for physical and chemical crosslinking, respectively, to obtain the double cross-linked bioplastic film. Compared with pure TEMPO-α-1,3-glucan oxidation complex or other types of polymer films, double cross-linked biological films

showed stronger thermal stability (lower thermal degradation efficiency) and mechanical strength (tensile strength 270MPa, Young's modulus 23GPa).

Conflict of Interest

The authors have no conflicts of interest.

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